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Dietary Linolenic and Docosahexaenoic Acid Influence the Docosahexaenoic Acid Content of Brain Membrane Lipids and Function in Developing Rats

By

Raffick Amid Razzakk Bowen ©

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in

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Raffick Bowen

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April, 2001

It is only through hard work that I can give an impression of ease and simplicity.
I must strive to erase all traces of effort and to reach clarity and purity.

-Henri Matisse

Always choose the unknown and go headlong.
Even if you suffer, it is worth it – it always pays.
You always come out of it more grown up, more mature, more intelligent.

-Osho

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Dietary Linolenic and Docosahexaenoic Acid Influence the Docosahexaenoic Acid Content of Brain Membrane Lipids and Function in Developing Rats** submitted by **Raffick Amid Razzakk Bowen** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Nutrition and Metabolism.**

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This thesis is respectfully dedicated to the health of all children and to the men and women who have spent their lives understanding and improving maternal and child health through nutrition.

ABSTRACT

One of the debates in infant nutrition concerns whether or not dietary C18:3n-3 can provide for accretion of C22:6n-3 in neonatal tissues. The functional significance of C22:6n-3 in brain has not been fully elucidated. The objectives of this study were to investigate if: (1) increasing maternal dietary linolenic acid (C18:3n-3) increases the docosahexaenoic acid (C22:6n-3) content in neuronal cell phospholipids in two-week-old rat pups; (2) increasing maternal dietary C18:3n-3 increases the C18:3n-3 and C22:6n-3 content of different tissues of two-week-old rat pups; (3) maternal dietary C22:6n-3 is more effective than high C18:3n-3 at increasing the C22:6n-3 content in neuronal and glial cell phospholipids in rat pups at two weeks of age; (4) maternal dietary C20:4n-6 and C22:6n-3 increases synaptic plasma membrane (SPM) phospholipid and cholesterol content, SPM phospholipid C20:4n-6 and C22:6n-3 content, and SPM sodium-potassium adenosine triphosphatase (Na, K-ATPase) activity in rat pups at two and five weeks of age; (5) C20:4n-6 and C22:6n-3 are present in gangliosides of SPM; (6) C20:4n-6 and C22:6n-3 content in SPM gangliosides can be altered by diet fat; and (7) alkaline ceramidase (CDase) activity is present in the SPM in two-week-old rat pups. The stomach fatty acid composition of the rat pups reflected the diets of their respective dams. Objective (1) established that phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) of neuronal cells showed no significant increase in C22:6n-3 content with high levels of C18:3n-3 in maternal diet; objective (2) showed that C18:3n-3 content in the whole body, brain, liver, skin, epididymal fat pads, and muscles was significantly greater in rat pups fed high compared to low C18:3n-3 diet but the C22:6n-3 content in these tissues was not quantitatively different; objective (3) showed that the

C22:6n-3 content in PE and PS of both neuronal and glial cell was higher when rat pups were fed C22:6n-3 compared C18:3n-3 diet ($p<0.05$); and objective (4) demonstrated that rats fed a maternal diet with C20:4n-6 and C22:6n-3 from two to five weeks of age: a) does not increase SPM cholesterol and phospholipid content, b) increases the C20:4n-6 (mainly PS) and C22:6n-3 content of SPM phospholipids, c) increases the SPM Na, K-ATPase Vmax (activity) (~2-fold) but not Km compared to a low C18:3n-3 diet; objective (5) showed by gas-liquid chromatography (GC) and GC-mass spectrometry that C20:4n-6 and C22:6n-3 are present in gangliosides of SPM of two-week-old rats; objective (6) suggest that the C20:4n-6 and C22:6n-3 content of SPM gangliosides of two-week-old rats can be altered by diet fat; and objective (7) showed that alkaline CDase activity is present in the rat SPM and is approximately 15-fold greater than brain homogenate.

It is concluded that dietary C22:6n-3 but not C18:3n-3 supports accretion of C22:6n-3 in neonatal tissues and that increasing the C22:6n-3 content of brain membrane phospholipid increases the activity of SPM Na, K-ATPase. It is also concluded that the presence of alkaline CDase activity in the SPM with the changes in C20:4n-6 and C22:6n-3 content of SPM gangliosides by diet fat may alter the activity of SPM alkaline CDase. Thus, dietary C18:3n-3 and C22:6n-3 can influence the structure and function of brain membrane lipids. This study supports the rationale for providing C20:4n-6 and C22:6n-3 in infant formulas.

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ABBREVIATIONS

AA	Arachidonic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BBM	Brush border membrane
C2	Two carbons
C6	Six carbons
CAPK	Ceramide activated protein kinase
CAPP	Ceramide activated protein phosphatase
CDase	Ceramidase
cDNA	Complementary deoxyribonucleic acid
Cer	Ceramide
CDP	Cytidine diphosphate
CMP	Cytidine monophosphate
CoA	Coenzyme A
CTP	Cytidine triphosphate
Da	Dalton
DHA	Docosahexaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EPG	Ethanolamine glycerophospholipid
ERK	Extracellular-regulated kinase
g	Gram
Gal	Galactose
GalNac	N-acetylgalactosamine
GD1a	Ganglioside (disialic acid; “a” sialo-isomer)
GD1b	Ganglioside (disialic acid; “b” sialo-isomer)
GLC	Gas-liquid chromatography
GM1a	Ganglioside (monosialic acid; “a” sialo-isomer)

GT1b	Ganglioside (trisialic acid; "b" sialo-isomer)
hr	Hour
HDL	High density lipoprotein
HMG-CoA	β-hydroxy-β-methyl-glutaryl-CoA
HPH	Hyperphenylalanemic
HPTLC	High performance thin-layer chromatography
IFN	Interferon
IL	Interleukin
K	Potassium
kb	kilobase
kDa	Kilo-Dalton
Km	Michaelis constant
LA	Linoleic acid
LCP	Long-chain polyenes (20-22 carbon atoms)
LCPUFA	Long-chain polyunsaturated fatty acid (20-22 carbon atoms)
LDL	Low density lipoprotein
LNA	Linolenic acid
M	Molar concentration
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated extracellular-regulated kinase-activating kinase
mg	Milligram, 10^{-3} gram
min	Minute
mL	Milliliter, 10^{-3} litre
mM	Millimolar concentration
mmol	Millimole
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acid
N-3	Omega-3

N-6	Omega-6
Na	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
NANA	N-acetylneuraminic acid
NeuAc	Neuraminic acid
PAH	phenylalanine hydroxylase
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine methyl transferase
pH	measure of acidity; = -log $[H^+]$
PHE	phenylalanine
PI	Phosphatidylinositol
PKU	Phenylketonuria
PKC	Protein kinase C
PL	Phospholipids
PLD	Phospholipase D
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
SAP	Sphingolipid activator proteins
SAT	Sialic acid transferase
SDS	Sodium dodecyl sulfate
SM	Sphingomyelin
SPM	Synaptic plasma membrane
SPP	Sphingosine-1-phosphate
SPT	Serine palmitoyl transferase
TLC	Thin-layer chromatography
TNF	Tumor necrosis factor
TPL	Total phospholipids

UDP	Uridine diphosphate
UV	Ultraviolet
VLCFA	Very long-chain fatty acid (> 24 carbon atoms)
Vmax	Maximal velocity
µg	Microgram, 10^{-6} gram
µL	Microlitre, 10^{-6} litre
µmol	Micromole, 10^{-6} µmol

CHAPTER I. LITERATURE REVIEW

A. INTRODUCTION

Lipids provide most of the dietary energy and constitute the major energy store in infants and children. Research over the past three decades has shown that there is interest in the quality of dietary lipids supplied during fetal and neonatal periods when there is rapid development of the nervous system.

Dietary fatty acid, particularly, linoleic (C18:2n-6) and linolenic (C18:3n-3) acid have received most of the attention in studies of the effects of dietary lipids on the central nervous system. The reason is that C18:2n-6 and C18:3n-3 cannot be synthesized by mammals but must be supplied in the diet. These two fatty acids are precursors of two series of long-chain polyunsaturated fatty acids (PUFAs), arachidonic (C20:4n-6) and docosahexaenoic (C22:6n-3) acid which constitute a large portion of the fatty acids in brain. Insufficient C20:4n-6 and/or C22:6n-3 delays growth and development of the brain and this may result in reversible and/or irreversible damage to the structure and function of the central nervous system.

In infant nutrition, human breast milk is the gold standard. It is clear that infant formulas do not meet all the qualities of mother's milk. Beside the psychological advantages of "bonding" through continuous breast-feeding or immunological factors, it is evident that the nutritional aspects of formulas do not contain all components of human milk such as enzymes, immune compounds, hormones, or growth factors. Improvements in the separation of fatty acids by gas-liquid chromatography (ie. capillary vs. packed columns) over the last 20 years have allowed scientists to study in greater detail the fatty acid composition of human milk and develop new infant formulas.

In North America, infant formulas generally derive their lipid composition from vegetable and animal oils and thus do not contain C20:4n-6 and C22:6n-3 as found in human milk. The absence of C20:4n-6 and C22:6n-3 in infant formulas compared to human milk has been shown over time to decrease brain membrane phospholipid C20:4n-6 and C22:6n-3 content (Farquarson et al., 1992; Makrides et al., 1994). This suggests that C18:2n-6 and C18:3n-3 present in infant formulas is inadequate for optimal nutrition in early life. In fact, the in vivo biosynthesis of C20:4n-6 and C22:6n-3 may be limited in newborns (Clandinin et al., 1980a; Salem et al, 1996; reviewed by Innis et al, 1999).

This chapter will review literature on the importance of brain growth and development; brain membrane lipids, in particular, phospholipids and gangliosides; and the effects of dietary n-6 and n-3 fatty acids on brain membrane structure and function. This information will be needed to support the rationale to be developed in subsequent chapters of this thesis.

B. BRAIN

1. Neuronal and Glial Cell Structure and Function

a) Neuronal Cells

The neuronal cell is the unit of structure and function of the nervous system, composed of a cell body and one or more processes (Figure 1.1). Neuronal cells are classified on a morphologic basis according to the number of their processes or upon the length of the axon (reviewed by Siegel et al., 1981).

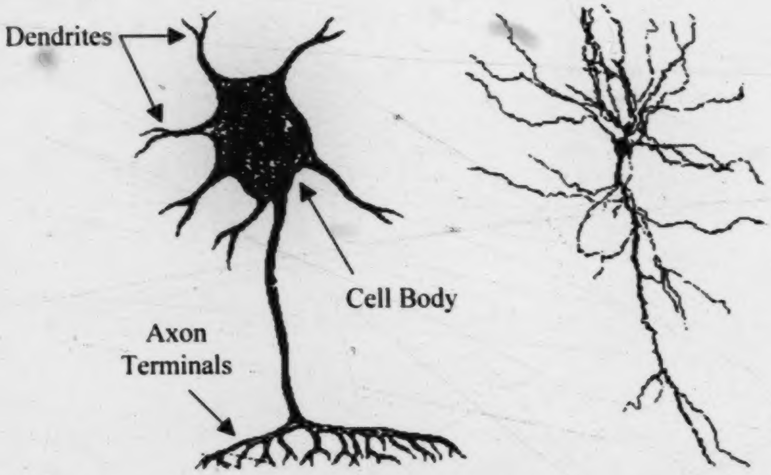


Figure 1.1 Structure of Neuronal Cell

(Adapted from Siegel et al, 1981)

i) Classification Based on Number of Processes

Neuronal cells may be unipolar, bipolar, or multipolar. Neuronal cells that have one, two, and more than two processes are called unipolar, bipolar, and multipolar neurons, respectively. Unipolar neuronal cells (sensory) are found almost exclusively in the peripheral nervous system (reviewed by Siegel et al., 1981). Bipolar neuronal cells have a dendrite and an axon attached to opposite ends of the cell body (reviewed by Siegel et al., 1981). Bipolar neuronal cells are found in the retina, in cochlea and vestibular ganglion, and some places in the central nervous system (reviewed by Siegel et al., 1981). Multipolar neuronal cells have only one axon, but several dendrites, each of which may give rise to secondary branches (reviewed by Siegel et al., 1981). Multipolar neuronal cells comprise the bulk of the neuronal cells whose cell bodies lie within the central nervous system (reviewed by Siegel et al., 1981).

ii) Classification Based on Axon Length

Morphologically, these neuronal cells are all of the multipolar type. Golgi type I neuronal cells have long axons, most of which leave the central nervous system via ventral root of spinal nerves (reviewed by Siegel et al., 1981). Golgi type II neuronal cells have short axons, all of which stay in the central nervous system (reviewed by Siegel et al., 1981). The majority of interneurons are of this type (reviewed by Siegel et al., 1981).

The major function of the neuronal cell is to help in the processing of incoming information so that appropriate motor responses can occur (reviewed by Siegel et al., 1981). The neuronal cell uses dendrites and axons to achieve these responses (reviewed by Siegel et al., 1981). Dendrites receive messages from the sense organs or other neuronal cell and carry them to the cell body (reviewed by Siegel et al., 1981). Axons, in turn, carry the resultant messages from the cell body to the other neuronal cells until an effector organ is reached (reviewed by Siegel et al., 1981). Hence, dendrites, cell body, and axons are crucial for neuronal cells to process and establish an appropriate response.

b) Glial Cells

In the nervous system, glial cells support neuronal cell function. There are four types of glial cells: astrocytes, oligodendrocytes, ependymal, and microglia (Figure 1.2;

reviewed by Siegel et al., 1981). Astrocytes are found throughout the central nervous system (reviewed by Siegel et al., 1981). Astrocytes are responsible for the blood-brain barrier, which prevents certain substances to pass from blood to the brain and neuronal cell tissue repair after brain injury (reviewed by Siegel et al., 1981). Oligodendrocytes are found in gray and white matter of the central nervous system (Siegel et al., 1981). Oligodendrocytes appear to hold gray matter together, whereas, in white matter, they are responsible for the myelin sheath around the axons (reviewed by Siegel et al., 1981). Ependymal cells line inner surface of the brain, in the ventricles (reviewed by Siegel et al., 1981). No physiological role has been assigned to these cells, however, the cilia present on the surface of these cells may be involved in propulsion of cerebrospinal fluid within the ventricles (reviewed by Siegel et al., 1981). Microglia, unlike astrocytes and oligodendrocytes are highly mobile (reviewed by Siegel et al., 1981). Microglia serve as macrophages to remove debris from the nervous system (reviewed by Siegel et al., 1981).

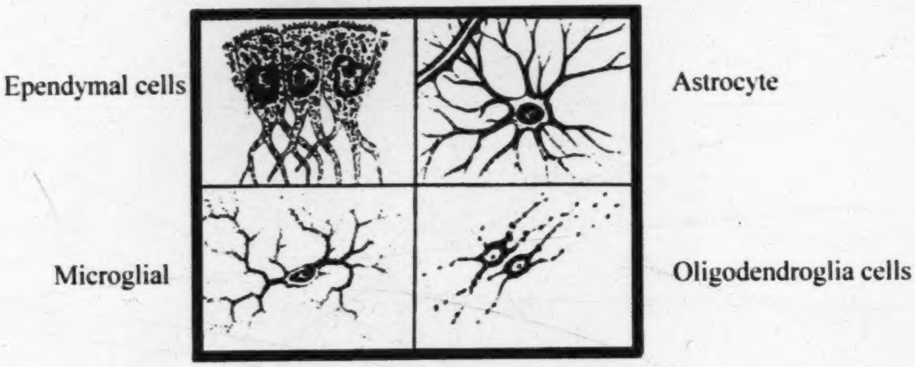


Figure 1.2 Structure of Glial Cells
(Adapted from Thompson et al., 1993)

2. Development

The human brain follows a general pattern of development, beginning as a neural tube and gradually acquiring the features of the adult brain (reviewed by Cowan, 1979). Eight major stages can be identified in the development of any part of the brain. In order of appearance, they are: (1) the induction of the neural plate, (2) the proliferation of cells, (3) the migration of cells from the region in which they are generated to the place where they finally reside, (4) the aggregation of cells to form identifiable parts of the brain, (5) the formation of connection with other neuronal cells (synaptogenesis), (6) the selective death of certain cells, (7) the elimination of some connections that were initially formed and the stabilization of others, (8) the myelination of axons by oligodendrocytes entering the brain.

a) Neuronal Induction

During embryogenesis, the basic structure of the nervous system develops by formation of the primitive streak during blastula stage (reviewed by Balinsky, 1975). This primitive streak separates the mesoderm from the ectoderm and endoderm (reviewed by Balinsky, 1975). The transformation of the ectoderm into neural plate is induced by the underlying mesoderm (Spemann, 1938). The inductive process may vary in different regions of the neural plate because brain stem and spinal cord arise under the chordal mesoderm whereas the forebrain arises under the influence of prechordal mesoderm (Nieuwkoop, 1952; Sidman & Rakic, 1982; Eyal-Giladi, 1984). The neural tube is formed by folding of the neural plate along the rostral-caudal axis, a process known as neuralation (O'Rahilly & Gardner, 1979). This process begins toward the end of the third embryonic week in humans (reviewed by Lou, 1982) and in rats (reviewed by Herschkowitz, 1989) between the first and second day of gestation. As neuralation progresses, the sequential association of the mesoderm and ectoderm results in induction of brain regions (forebrain, midbrain, and hindbrain) and spinal cord structures (reviewed by Lou, 1982).

At the time of neural tube closure, cells at the edge of the neural plate separate from neuroepithelium and migrate into the extracellular matrix to become neural crest cells (Angevine & Sidman, 1961). Following migration of neural crest cells to their final locations, these neural crest cells differentiate into neuronal and glial cells of dorsal roots and autonomic ganglia; neural crest cells provide the gastrointestinal tract and sensory ganglia of the cranial nerves and melanocytes (Brown et al., 1991). The neuroepithelium, or ventricular zone, that contains pseudostratified columnar epithelial cells that line the wall of the neural tube are actively proliferating (Sauer, 1935). As the cells within the neuroepithelium become postmitotic (never divide again) and migrate outward and differentiate, the neural tube becomes thicker from the outer surface and the ventricular zone becomes defined (Sidman et al., 1959; Watterson, 1965). The cells of the neuroepithelium differentiate into two cell types of the nervous system: the neurons, which form the functional unit of the nervous system, and the glia cells which provides various types of support functions to neuronal cells and are important in myelin function (Sidman & Rakic, 1982).

b) Neurogenesis

Neuronal cell proliferation occurs within the neuroepithelium lining the ventricular zone of the neural tube (Sauer & Chittenden, 1959; Sidman et al., 1959; Fujita, 1962). Neuronal proliferation begins around fourteenth week of gestation and is completed by the twenty-fifth week when an adult number of neuronal cells is present (Dobbing & Sands, 1970; reviewed by Lou, 1982). In rats, the developmental events occur at eighteen days of gestation and is completed by twenty days gestation (reviewed by Lou, 1982). The period of neuronal cell proliferation may well be extended over longer periods of time. In rats, it has been shown that the number of neuronal cells in the hippocampus increase until one year of age (Bayer et al., 1985). These results suggest that also in humans the number of neuronal cells in some regions may increase postnatally.

c) Gliogenesis

Glial cells tend to originate after neuronal cells in any particular region of the brain (Ichikawa & Hirata, 1982). Glial cells differ from neural cell formation. Firstly, cells that produce glia lie outside the neuroepithelium, at or near the site where they will be located in adults (Bayer, 1985). Secondly, glial cell production continues throughout life (Jacobson, 1970). Gliogenesis is a postnatal event after term gestation (Das, 1977), but in some brain regions it has been detected before birth (Das, 1977; Rodier, 1980). Early gliogenesis is completed by the fifteenth week of gestation in human and the sixteenth day in rats (reviewed by Morgane et. al., 1993), and thirdly, damage to glial cells is not permanent. Glial cells can recover from low level of irradiation and brain injury (Bayer & Altman, 1975).

d) Neuronal Cell Migration

Since neuronal cells of the central nervous system are generated close to or at the neuroepithelium of the neural tube, they must subsequently undergo migration past other cells before reaching their definitive locations where they will reside for life (Sidman & Rakic, 1973). The peak time for this occurrence in humans is the third to fifth month of gestation, although it has been detected in certain brain regions as early as the second month and slightly after the fifth month (Sidman & Rakic, 1973). In the 1970s, it became clear that neuronal cells migrate radially outward from the ventricular layers along surface of radial glial cells (Rakic, 1977). Evidence for this relationship between neuronal cells from radial glial cells came from the *Weaver* mouse (Rakic & Sidman, 1973). In this mouse model, Bergmann glial cells in the cerebellar cortex degenerated at an early stage and failed to migrate granule cells from external to the internal granule layer (Rakic & Sidman, 1973). These findings suggested that granule cells failed to migrate due to the lack of glial cell guidance (Rakic & Sidman, 1973). In a comprehensive series of experiments, Rakic (1971a and 1971b) studied the development of the cerebral cortex, hippocampus, and cerebellum in monkeys and humans (reviewed by Rakic 1988a, 1988b, 1990, 1995). The formation of the various cell types and their migration to their final destinations have been followed by light and electron microscopy and by labeling of the glial cells with specific antibodies (Rakic, 1971a and 1971b). Similar experiments in mice (Luskin, 1988) were performed with recombinant retroviruses encoding a marker gene to infect early neuronal and glial cell progenitors (reviewed by Luskin, 1994; Goldman & Luskin, 1998; and Pavlath & Luskin, 1999). The results of these studies demonstrated that neuronal cells moved along the glial cell processes during development (Rakic, 1971a and 1971b; Luskin et al., 1988; reviewed by Rakic 1988a, 1988b, 1990, and 1995).Thus, glial cells are needed for neuronal cell migration.

e) Neuronal Cell Aggregation

Neuronal cell migration ceases in brain when the cells reach their destination and selectively aggregate to form a group or a cortical layer (Sidman & Rakic, 1973). The underlying mechanism of aggregation is not yet known but it has been suggested that adhesion molecules may play a major role in the aggregation process (reviewed by

Nybrue & Bock, 1989). Cell adhesion molecules are proteins that mediate cellular interaction in the embryo. Cell adhesion molecules function in the pattern formation and morphogenesis of tissues (reviewed by Edelman, 1989). Neuronal cell adhesion molecules, which are expressed by all major cell types in the nervous system could be responsible for neuronal cell migration (reviewed by Edelman, 1989).

f) Axon and Dendrite Outgrowth

As neuronal cells migrate along radial glia, many neuronal cells extend processes that are recognizable as axons (reviewed by Bray & Hollenbeck, 1988). The tips of each process are composed of a specialized motile structure, a growth cone (reviewed by Bray & Hollenbeck, 1988 and Jacobson, 1991). Growth cones are constantly moving by local extension and retractions (reviewed by Jacobson, 1991). When in contact with appropriate substrate, growth cones crawl forward and leave behind the elongating axon (reviewed by Bray & Hollenbeck, 1988). Axon outgrowth is a prenatal and a postnatal event in both the human and rats (reviewed by Herschkowitz, 1989). The mechanism involved in the growth and guidance of axons appear to be specific and non-specific (reviewed by Herschkowitz, 1989).

The migrating neuronal cells begin to sprout dendrites as soon as they arrive at the cortical plate (Juraska & Fifkova, 1979). The apical and basilar dendrites are formed first which is followed by oblique branches off the apical dendrites (Juraska & Fifkova, 1979). Golgi-Cox studies of cortical neuronal cells show that the complexity of dendrites continues to increase for at least twenty-four months postnatally (Conel, 1939). There are regional differences in rate of dendritic growth (Purpura, 1975; Goldman & Rakic, 1983).

g) Synaptogenesis

Synaptogenesis is the contact between axons and target cells. Synaptogenesis starts before neurogenesis is completed (reviewed by Jacobson, 1978). Synaptic formation differs appreciably among brain regions in the human brain (reviewed by Jacobson, 1978). Synapses appear in the human cerebral cortex as early as the third month of gestation (Molliver et al., 1973). The number of synapses increases coincident with the elaboration of dendritic spines, which are the primary sites onto which synaptic contacts are made (Paldino & Purpura, 1979a and 1979b). Quantitative determination by electron microscopy have demonstrated that the number of synapses increases until eight months postnatally in the striate cortex and until twenty-four months after birth in the frontal cortex (Huttenlocher, 1979; Huttenlocher et al., 1982; Huttenlocher & de Courten, 1987). After reaching these peak values, a slight reduction in number occurs over the course of many months (Huttenlocher & de Courten, 1987). Although patterns of neuronal cell connection are initiated by directed axon outgrowth, ultimate cortical organization is effected by specific neuronal cell interaction (Sperry, 1963; reviewed by Edelman, 1983). These interactions appear to be mediated by molecules on the surface of neuronal cells and neuronal cell processes that result in the selective linking of axons to certain neurons by specific chemical affinity (Sperry, 1963). Neuronal cell adhesion molecules have been shown to promote cell-to-cell interactions among neuronal cells (reviewed by Edelman, 1983) but the final pattern of synaptic connectivity appears to be shaped by events such as competition for trophic factors and modulators that affect neuronal cell activity or target cells (Purves & Lichtman, 1985; Purves, 1988).

Following synaptogenesis, a period of synaptic refinement occurs which is fundamental for normal neuronal cell function. Synaptic refinement decreases the number of inputs per cell by eliminating weaker connections by apoptosis (programmed cell death) and strengthening of the remaining connection through successful competition for trophic substances (Lictman & Purves, 1980; Purves & Lictman, 1980). Therefore, apoptosis and synapse refinement thus follows as the final stage of brain morphogenesis (Bayer, 1985).

h) Myelination

Undifferentiated oligodendrocytes arise within the ventricular zone and migrate in the cerebral wall (Oksche, 1968; reviewed by Colello & Pott, 1997). As these precursors migrate in the brain, they respond to local influences of other neuronal and glial cells by proliferation and differentiation (Noble, 1986). The oligodendrocytes then align along axons and elaborate cell processes that ultimately form myelin sheath (Peters, 1969; reviewed by Colello & Pott, 1997). In the central nervous system, oligodendrocytes may produce myelin for up to forty parallel axon segments (Peters, 1964). Myelination exhibits pronounced regional variation (Yakovlev & Leaours, 1967; reviewed by Colello & Pott, 1997). In human, myelination begins in the prenatal period and continues into adult life (reviewed by Herschkowitz, 1988 and Colello & Pott, 1997).

3. Critical Periods

The cells, regions, and various structures of the brain do not develop uniformly as in other tissues and organs (Dobbing & Sands, 1979; Dobbing, 1990). There are characteristic, well-defined stages of growth that occur anatomically and biochemically (Gottlieb et al., 1977; Albers, 1985) and result in significant growth spurts or critical periods in fetal and neonatal life. Critical periods are defined as specific times of increased metabolic activity during which a particular developmental process predominates (Dobbing & Sands, 1979). In view of the complexity of the brain, it is conceivable that normal brain development could be disrupted by any number of events, particularly during the critical periods of brain growth and development. Influences of exogenous factors, such as, the amount or type of dietary n-6 and n-3 fatty acids on brain membrane lipid composition during development could possibly alter brain structure and function (e.g. sodium-potassium adenosine triphosphatase transport activities) (Foot et al., 1982; reviewed by Hargreaves & Clandinin, 1990; Clandinin et al., 1997; Clandinin, 1999).

C. SODIUM-POTASSIUM ADENOSINE TRIPHOSPHATASE

1. Biological Functions

The sodium-potassium adenosine triphosphatase (Na, K-ATPase), or sodium pump, is a membrane bound protein that establishes and maintains the high internal potassium ion and low internal sodium ion concentration typical of most animal cells (reviewed by Wallick, 1979). The Na, K-ATPase transfers chemical energy of hydrolysis of adenosine triphosphate (ATP) to potential energy of electrochemical ion gradients found by sodium ions and potassium ions across the cell membrane (reviewed by Wallick, 1979). Na, K-ATPase transports three sodium ions out in exchange for two potassium ions that are taken into the cell (reviewed by Wallick, 1979). The electrochemical gradient that Na, K-ATPase generates is for a large number of basic and specialized cellular functions like regulation of cell volume, the resting membrane potential of tissues, and excitable properties of muscle and nerve cells (reviewed by Jorgensen, 1990). A variety of secondary active systems that transport ions (calcium, hydrogen, chloride, sulphate, and phosphate) or nutrients (glucose and amino acids) across the cell membrane are driven by the inwardly directed electrochemical gradient for sodium ions (reviewed by Skou & Esmann, 1992 and Glynn, 1993).

2. Structure

Na, K-ATPase is an oligomeric integral membrane protein that is composed of stoichiometric amounts of two major polypeptides, the alpha and beta subunits (reviewed by Jorgensen & Anderson, 1988 and Lingrel, 1992). The alpha-subunit is a multi-spanning membrane protein with a molecular mass of about 112 kDa (reviewed by Jorgensen & Anderson, 1988 and Lingrel, 1992). This subunit is responsible for both catalytic and transport functions of the enzyme (reviewed by Jorgensen & Anderson, 1988 and Lingrel, 1992). The alpha subunit comprises of a binding site for cations, adenosine triphosphate, and cardiac glycosides (i.e. ouabain, digoxin, or digitoxin; reviewed by Jorgensen & Anderson, 1988 and Lingrel, 1992). Three isoforms of the alpha-subunit (α_1, α_2, and α_3) appear to be expressed in rodent brains (Shull et al., 1986). The three isoforms from cDNA clones show about 85% sequence homology, with the most substantial differences occurring in the N-terminal region (Shull et al., 1986). The regions including the catalytic phosphorylation site are identical for a length of 85 amino acid residues, and there are major hydrophobic domains with 94-96% homology between the three isoforms (Shull et al., 1986). The three alpha-subunit isoforms are expressed to varying extents at different stages of development (Shull et al., 1986). There is evidence that at least five different genes encode for the alpha-subunit (Shull et al., 1986). Transcriptions of the α_1-subunit genes were detected in all tissues, whereas α_2 and α_3 subunits mRNA were expressed predominately in brain (Emanuel et al., 1987).

The smaller constituent, beta subunit mainly β_1 and β_2, is a polypeptide that crosses the membrane once and, depending on the degree of glycosylation in different tissues, has a molecular weight between 40 and 60 kDa (Blanco et al., 1994). The subunit was for a long time believed not to participate directly in the catalytic cycle or binding of cardiac glycosides. Recently, it was demonstrated that assembly of a (alpha-beta) heterodimer is necessary for a stable and functionally competent configuration of the pump (Figure 1.3); in particular, the beta subunit is needed for the alpha subunit to exit from the endoplasmic reticulum and to acquire the correct configuration (Geering et al., 1989; Kawamura & Noguchi, 1991). The beta subunit appears to be involved in the occlusion of potassium ion and the modulation of the potassium and sodium ion affinity of the enzyme (Lutsenko & Kaplan, 1993). The amino acid sequence of the beta subunits has been detected from cDNA clones and the tissue distribution of the beta isoforms and the corresponding mRNA has been examined (Shull et al., 1986; Mercer et al., 1986; Young et al., 1987; Martin-Vasallo et al., 1989; Levensen, 1994). The β_1-subunit was composed of 304 amino acid residues, whereas the β_2-subunit had 290 residues (Levenson, 1994). The two beta-subunits had approximately 58% amino acid sequence homology (Levenson, 1994). The β-subunit gene encodes mRNA ranging in size from approximately 1.9 to 3.9 Kb (Levenson, 1994). Previous studies has shown that multiple beta subunits mRNAs are derived by alternative splicing and encodes a single β-subunit polypeptide (Young et al., 1987). The pattern of expression of beta-subunit mRNA is complex and tissue-specific (Mercer et al., 1986; Emanuel et al., 1987), but is distinct from the alpha-subunit mRNA (Mercer et al., 1986; Emanuel et al., 1987).

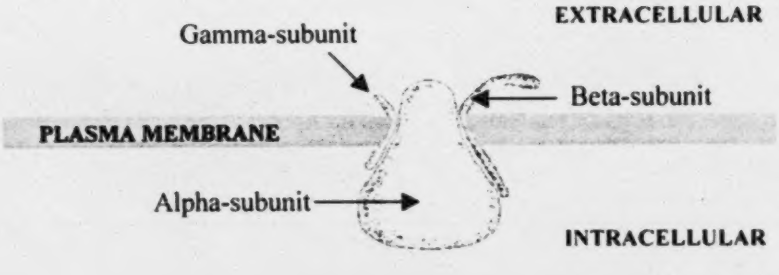


Figure 1.3 Structure of Na, K-ATPase

(Adapted from Mercer, 1999)

A third protein termed the gamma-subunit with a molecular mass between 8 to 14 kDa has been detected as part of the native enzyme in purified separations (Reeves et al., 1980). The gamma subunit is expressed primarily in renal tissues (Therien et al., 1997). Expression studies have shown that the gamma subunit is not required for normal Na, K-ATPase activity (Hardwicke & Freytag, 1981; Detomasso et al., 1993). However, recently it was shown that the gamma subunit can modify the voltage dependence of potassium ion activation when expressed in *Xenopus* oocytes (Beguin et al., 1997). It appears that the gamma subunit can stabilize the E1 conformation of the enzyme (Therien et al., 1997). Although there is increasing evidence that the gamma subunit can modify Na, K-ATPase function, the exact role of the subunit in Na, K-ATPase function awaits further investigation. Further studies have shown that the gamma-subunit of Na, K-ATPase consists of 58 amino acids with a molecular mass of approximately 6.5-7.0 kDa (Mercer et al., 1993). cDNAs for the human (Kim et al., 1997) and *X. laevis* (Beguin et al., 1997) gamma subunits have been cloned and sequenced. Amino acid sequence comparisons shown strong homology (~75%) among different species, which is further increased to 93% when only mammalian sequences are compared (Kuster et al., 2000).

3. Cellular Localization

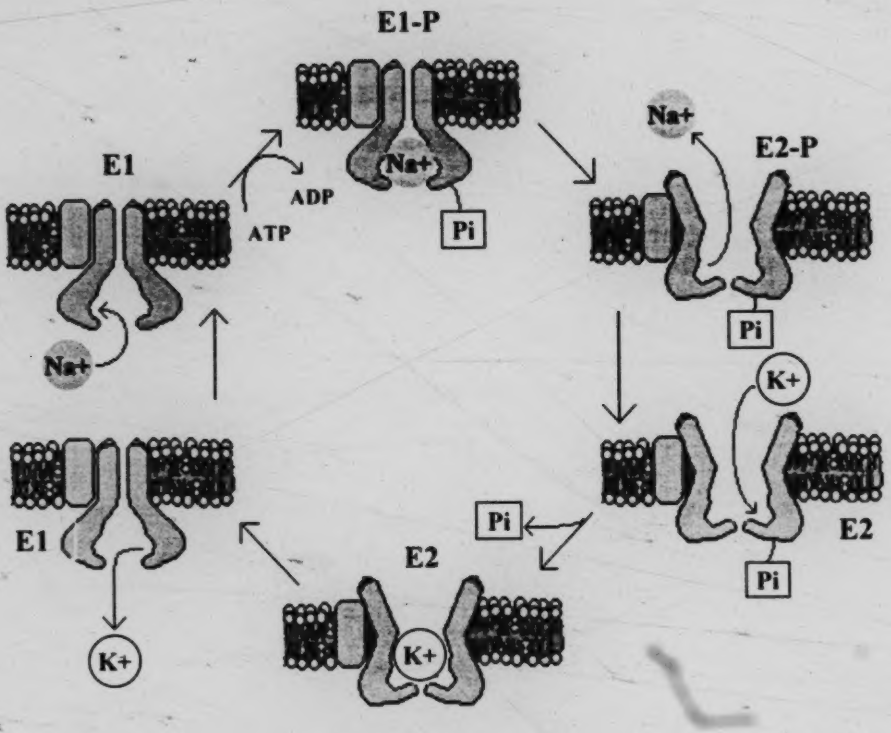
In the nervous system, Na, K-ATPase has been found to be associated with both neuronal and glial cells (Cummins & Hyden, 1962). Na, K-ATPase is found to be particularly most active in the gray matter of the nervous tissue (Bonting et al., 1962). The gray matter exhibits approximately fivefold more enzyme activity than the white matter (Bonting et al., 1962). Hosie (1965) and Albers et al. (1965) have shown that an enriched preparation of Na, K-ATPase can be obtained from nerve endings fraction of brain (synaptic plasma membrane; SPM). Kurokawa et al. (1965) confirmed by sucrose gradient technique that there was indeed high specific activity of Na, K-ATPase in nerve fraction of brain.

4. Transport Cycle

Na, K-ATPase belongs to a class of P-type ATPase that are responsible for active transport of a variety of cation across cell membranes (Sachsand & Munson, 1991; Apell, 1997; Holmgren et al., 2000). The P-type description refers to the unique characteristic of ATPase in forming a transient, phosphorylated aspartyl residue during the catalytic cycle (Sachsand & Munson, 1991; Apell, 1997; Holmgren et al., 2000). Accompanying the phosphorylation-dephosphorylation process, the P-type ATPase bind, occlude, and transport ions by cycling between two different cation-dependent conformations, called E1 and E2 (Repke & Schon, 1992; Apell, 1997; Holmgren et al., 2000). The precise molecular mechanisms that couple the hydrolysis of adenosine triphosphate to the conformational changes and the translocation of ions remain unknown.

The description of the Na, K-ATPase transport is based on the hypothesis proposed by Albers (1967) and Post et al. (1969) (Figure 1.4). The Na, K-ATPase undergoes a sequence of transition between the E1 conformation with inward facing cation binding sites and high affinity for sodium ion and the E2 conformation with outward facing cation binding sites and high affinity for potassium ion (Albers, 1967; Post et al., 1969). Transitions between these two conformations are induced by the phosphorylation (E1-P and E2-P; Figure 1.4) -dephosphorylation (E1 and E2; Figure 1.4) reactions (Albers, 1967; Post et al., 1969). In the E1 Na, K-ATPase form, three intracellular sodium ions become bound (Albers, 1967; Post et al., 1969). Hydrolysis of adenosine triphosphate and phosphorylation of the protein (E1-P; Figure 1.4) leads to occlusion of the three sodium ions followed by a transition to the E2-P form (Albers, 1967; Post et al., 1969). Sodium ion is now released externally, and instead two potassium ions become bound; this leads to spontaneous dephosphorylation and occlusion of the potassium ions (E2; Figure 1.4; Albers, 1967; Post et al., 1969). Stimulated by adenosine triphosphate, a conformational change back to E1 form is induced, potassium is fed into the cytoplasm and the transport cycle is completed (Albers, 1967; Post et al., 1969).

The role of membrane lipids on the function of Na, K-ATPase is discussed on page 26.



The top is extracellular.
The bottom is intracellular.

Figure 1.4 Na, K-ATPase Transport Cycle
(Adapted from Dalton, 1999)

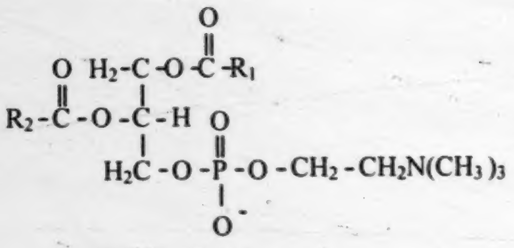
5. Significance in Brain

In brain, at least 40% of the energy released by respiration is required by the Na, K-ATPase in order to maintain the ionic gradients of sodium and potassium across cell membranes, even under resting conditions (Whittam, 1962; Ritchie, 1980; Astrop et al., 1981; Yarowasky & Ingvar, 1981; Hansen, 1985). The Na, K-ATPase has a critical role in the functioning of the brain. The activity of Na, K-ATPase is present in high concentrations in both neuronal and glial cells (reviewed by Sweadner & Goldin, 1980). In addition, to maintaining the ion gradients required for nerve impulses, the enzyme is implicated in the indirect modulation of synaptic action (reviewed by Sweadner & Goldin, 1980). When the enzyme is very active, it hyperpolarizes the nerve membrane altering the threshold for synaptic action (reviewed by Albers, 1967). When Na, K-ATPase activity is low, sodium ions accumulate in the nerve terminals and are exchanged for extracellular calcium by sodium / calcium exchange system (Goddard & Robinson, 1976; Akerman & Nicholls, 1981; DiPolo & Beauge, 1983). The elevated intraterminal calcium concentration then facilitates the release of neurotransmitters during subsequent impulses, since the release of neurotransmitter is calcium dependant (reviewed by Lees, 1991). These processes may operate to different extents in the presynaptic and postsynaptic cells (reviewed by Lees, 1991). Also, it has been reported that the activity of Na, K-ATPase is increased by certain neurotransmitters (reviewed by Lees, 1991). In glial cells, the Na, K-ATPase plays a critical part by taking up the potassium released by neurons during periods of intense activity, when potassium reaches extracellular concentrations high enough to depolarize the nerve membrane (reviewed by Lees, 1991). The failure of this process can result in epileptic seizure (Hertz, 1978).

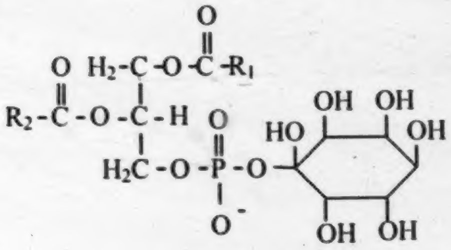
D. PHOSPHOLIPIDS

1. Structure

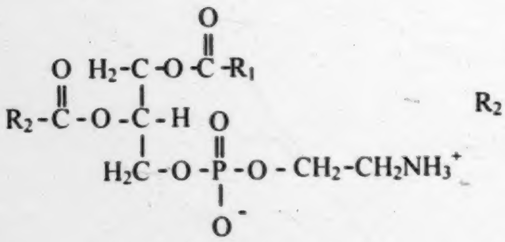
Phospholipids contain both fatty acids and phosphoric acid esterified to an alcohol (reviewed by Ramsey & Nicholas, 1972; Strickland, 1973; Porcellati et al., 1976). Polar head groups such as choline, ethanolamine, serine, and inositol may be esterified with the second acid group on the phosphate (Figure 1.5). Further diversity in phospholipids is provided by the variation in the fatty acids present with palmitic (C16:0) , stearic (C18:0), oleic (C18:1n-9) , C18:2n-6, C20:4n-6, and C22:6n-3 being most common. In general, the hydroxyl group at position-1 of the glycerol moiety of a PA is esterified with a saturated fatty acid and that at position-2 is esterified with an unsaturated fatty acid reflecting the specificity of the enzymes involved in the synthesis (Strickland, 1973; Porcellati et al., 1976). Regardless of structure, all phospholipids are markedly polar, possessing both a hydrophobic tail (two hydrocarbon chains) and a hydrophilic head (containing the phosphate group and the second alcohol which rotate away from the tail).



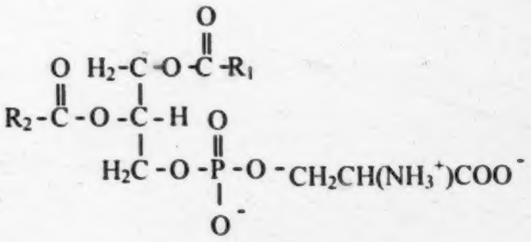
Phosphatidylcholine



Phosphatidylinositol



Phosphatidylethanolamine



Phosphatidylserine

R_1 and R_2 = Fatty acyl chains

Figure 1.5 Structure of Phospholipids

2. Role in Cell Membranes

Phospholipids along with cholesterol are the primary lipid constituents of membranes. The two opposing lipid leaflets make up the membrane bilayer which serves as a permeability barrier imparting cellular compartmentalization and support matrix for membrane-bound proteins (Singer & Nicholson, 1972). Biological membranes are fluid in nature which permits lateral diffusion of lipid and protein in the plane of the membrane (Coleman, 1973; Sun et al., 1983). The hydrophilic head group and hydrophobic fatty acyl tails make phospholipids amphipathic. In the membrane bilayer, phospholipids orient in such a way that their hydrophilic head groups face outward contacting the aqueous phase while the hydrophobic fatty acyl tails aggregate and exclude water (Singer & Nicholson, 1972).

3. Synthesis in Brain

Before the 1940's, it was generally assumed that once phospholipids are synthesized in the nervous system of mammals during growth and development they were static entities (Ansell & Spanner, 1961). The introduction of isotopes into research, especially [^{32}P] inorganic phosphate dispelled this assumption when it was shown that once [^{32}P] inorganic phosphate were available to the intracellular pool, they become rapidly incorporated into membrane phospholipids (Ansell & Spanner, 1967).

The pathways of phospholipid biosynthesis are similar among different tissues and organs (Ansell & Spanner, 1967). However, the central mechanism regulating the changing levels and composition of phospholipids during brain development are not well understood. Precursor availability and/or the levels and modulators of enzyme activity could be mechanisms that regulate phospholipid synthesis (Ansell & Spanner, 1967).

a) Phosphatidylcholine

Two pathways exist for the de novo synthesis of phosphatidylcholine (PC): (1) the cytidine diphosphate (CDP)-choline pathway which synthesizes PC from CDP-choline and diacylglycerol (DAG), and (2) the phosphatidylethanolamine methyl transferase (PEMT) pathway which converts phosphatidylethanolamine (PE) to PC.

i) CDP-Choline Pathway

When labeled choline was injected intracerebally into the adult rat and the specific radioactivity and time sequence of phosphorylcholine, CDP-choline, and PC were determined, it became apparent the cytidine route was the principal pathway for de novo biosynthesis of PC (Ansell & Spanner, 1968) (Figure 1.6). Choline derived mostly from diet or by synthesis in liver can enter the brain and be rapidly phosphorylated by choline kinase to form phosphorylcholine (McCaman, 1962). The structural gene for choline kinase has been cloned from yeast (Hosaka et al., 1989) and the cDNA from rat liver (Uchida & Yamashita, 1992). The conversion of phosphocholine into CDP-choline requires a cytidylyltransferase enzyme (Porcellati & Arienti, 1970). To date, cDNAs for cytidylyltransferase have been cloned from several mammals (humans, mouse, and chinese hamster) (Rutherford et al., 1993; Kalmar et al., 1994; Sweitzer & Kent, 1994). The deduced protein sequence of these enzymes is 95-99% identical to each other. The enzyme has a subunit molecular weight of 42 kDa, and the purified forms exists as a dimer (Cornell, 1989; Weinhold et al., 1989). The CDP-choline can then be transferred to DAG by CDP-choline:1,2-DAG choline transferase to form PC (McCaman & Cook, 1966). CDP-choline:1,2-DAG choline transferase has been cloned from yeast (Hjelmstad & Bell, 1990) but only partial purification of this enzyme from liver has been achieved so far (O & Choy, 1990). CDP-choline:1,2-DAG choline transferase from yeast has 407 amino acids with a predicted molecular weight of approximately 46 kDa (Hjelmstad and Bell, 1990). This enzyme has been shown to display its highest activity in vitro preparation of neonatal tissue and neuronal rich gray matter (McCaman & Cook, 1966). Thus, demonstrating the importance of this enzyme in neonatal neuronal PC biosynthesis.

ii) Phosphatidylethanolamine Methyltransferase

The transfer of methyl groups from S-adenosylmethionine to PE by methyltransferase is an alternative route for the formation of PC (Figure 1.6; Bremer et al., 1960). This methylation pathway is quantitatively significant only in liver where it may account for about 20-40% of the synthesis of PC (Sundler & Akesson, 1975). In all other tissues, including brain, the activity of the methylation enzyme(s) is very low (Sundler & Akesson, 1975). Sastry et al. (1985) suggested that two enzymes function in animal tissues for the methylation reaction, one transferring the first methyl group to PE, and a second enzyme catalyzing the second and third methylation. Pajares et al. (1984) purified methyltransferase from rat liver and found that this enzyme catalyzes all three methylation steps of PE synthesis. However, in *Saccharomyces* (Greenberg et al., 1983) and in *Neurospora* (Scarborough & Nyc, 1967), it appears that at least two enzymes are involved in the stepwise methylation sequence. Thus, it is possible that different tissue express different number of methyltransferase in the PEMT pathway. Recently, the murine gene that codes for PEMT was cloned and characterized (Walkey et al., 1996).

This gene predicts a 199 amino acid protein with a molecular weight of 22.5 kDa and has greater than 92% homology with rat PEMT cDNA (Walkey et al., 1996).

Diets containing different ratios of soybean and sunflower oil were mixed to produce a range of dietary n-6 to n-3 fatty acids ratios (Hargreaves & Clandinin, 1989). Resulting levels of C22:5n-6 found in microsomal membrane PE correlated with production of phosphatidylmethylethanolamine via the PEMT pathway, but was negatively correlated with CDP-choline pathway (Hargreaves & Clandinin, 1989). Thus, PC synthesis via PEMT and CDP-choline pathways responds to change in diet fat.

b) Phosphatidylethanolamine

i) CDP-Ethanolamine Pathway

The de novo synthesis of PE occurs via the CDP-ethanolamine pathway (Figure 1.6). The phosphorylation of ethanolamine by kinase is different from that of choline as seen by different requirements of kinase for magnesium and adenosine triphosphate (Spanner & Ansell, 1979). This suggests that two separate enzymes may be involved in choline or ethanolamine phosphorylation. The formation of CDP-ethanolamine by cytidine triphosphate (CTP):ethanolamine phosphate cytidylyltransferase seems to be the rate-limiting enzyme in the formation of PE (Porcellati et al., 1971). Nakashima et al (1997) reported the cloning of a human cDNA for CTP:ethanolamine phosphate cytidylyltransferase by complementation *in vivo* of a yeast mutant of which the CTP:ethanolamine phosphate cytidylyltransferase gene was disrupted. The deduced protein encoded by the cDNA consists of 389 amino acids with a calculated molecular mass of approximately 43.8 kDa (Nakashima et al., 1997). The combining of CDP-ethanolamine and DAG by CDP-ethanolamine:1,2-DAG ethanolamine phosphotransferase is the final step in PE formation (Ansell & Metcalfe, 1971). The DAG species containing PUFAs rather than monenoic, dienoic, or trienoic species seems to be the preferred substrates for PE synthesis (Holub, 1978). Complementation of *S. cerevisiae* mutants defective in CDP-ethanolamine:1,2-DAG ethanolamine phosphotransferase has resulted in the isolation of a gene encoding CDP-ethanolamine:1,2-DAG ethanolamine phosphotransferase activity (Hjelmstad & Bell, 1991). The derived amino acid sequence for the *S. cerevisiae* CDP-ethanolamine:1,2-

DAG ethanolamine phosphotransferase gene predicts a protein of 44.5 kDa containing seven membrane-spanning domains (Hjelmstad & Bell, 1991). Recently, CDP-ethanolamine:1,2-DAG ethanolamine phosphotransferase has been purified from bovine liver microsomes (Mancini et al., 1999). The isolated protein has a molecular mass of about 38 kDa (Mancini et al., 1999). Future research should provide amino acid sequence and gene cloning for this enzyme.

ii) Decarboxylation of Phosphatidylserine

PE formation occurs by decarboxylation of phosphatidylserine (PS; Figure 1.6). Bremer et al (1960), using radiolabeled L-[3-^{14}C] serine showed *in vivo* labeling of PE in liver. Wilson et al. (1995) who incubated labeled serine with liver mitochondria and brain homogenates also showed the appearance of the label in the ethanolamine moiety of PE. Dennis & Kennedy (1972) clearly demonstrated the decarboxylation of PS by a mitochondrial enzyme in liver, PS decarboxylase (Dennis & Kennedy, 1972). Experiments by Yavin & Ziegler (1977) established that about 13% of PE were formed via this route in differentiating cells of the cerebral hemisphere. The cDNA for the mammalian PS decarboxylase was cloned in somatic cell mutants (Kuge et al., 1991; Kuge et al., 1996). The full-length cDNA for PS decarboxylase was isolated by polymerase chain reaction methods and it encodes for a protein of 409 amino acids (Kuge et al., 1991; Kuge et al., 1996).

c) Phosphatidylinositol

Studies by Agranoff et al. (1958) and Paules & Kennedy (1958) lead to the discovery of CDP-DAG:myoinositol phosphatidyltransferase that catalyzes the synthesis of phosphatidylinositol (PI; Figure 1.6). CDP-DAG-myoinositol phosphatidyltransferase catalyzes the reaction of CDP-DAG with myoinositol (Agranoff et al., 1958; Paules & Kennedy, 1958). This enzyme appears to be located in the microsomal fraction of liver (Takenawa & Egawa, 1977) and brain (Ghalayini & Eichberg, 1985). The enzyme shows high selectivity for myoinositol (Benjamins & Agranoff, 1969). Unfortunately, no amino acid sequence information has yet been obtained for the mammalian enzyme. However, recently Tanaka et al (1996) has cloned the rat CDP-DAG:myoinositol phosphatidyltransferase cDNA by functional complementation of a *S. cerevisiae* CDP-DAG:myoinositol phosphatidyltransferase mutant, deficient in CDP-DAG:myoinositol phosphatidyltransferase activity. The cloned cDNA encodes a protein of 213 amino acids with a calculated molecular mass of approximately 24 kDa (Tanaka et al., 1996).

d) Interconversion or Base Exchange: Phosphatidylcholine, Phosphatidylethanolamine, and Phosphatidylserine Synthesis

It is known that base-exchange enzymes of the nervous system exist that are responsible for the energy-independent incorporation of choline, ethanolamine, and serine into membrane phospholipid with the formation of PC, PE, and PS, respectively (Figure 1.6; Porcellati et al., 1971; Kanfer, 1972). These enzymes are membrane-bound to the microsomal fraction (Porcelatti et al., 1971; Kanfer, 1972) and have a strict requirement for calcium (Buchanan & Kanfer, 1980). The serine-base exchange enzymes (PS synthase) seem to be the sole route for PS production in brain as well as other tissues and PE seems to be the preferred substrate for exchange (Yavin & Zeigler, 1977). PS synthase cDNA has been isolated from Chinese hamster ovaries (Kuge et al., 1991). It has been shown by immunoprecipitation that the PS synthase has an apparent molecular mass of 42 kDa (Kuge et al., 1991). Recently, partial human cDNA encoding PS synthase has been found (Kuge et al., 1997). The gene sequence is indicative of a protein of approximately 474 amino acids (Kuge et al., 1997).

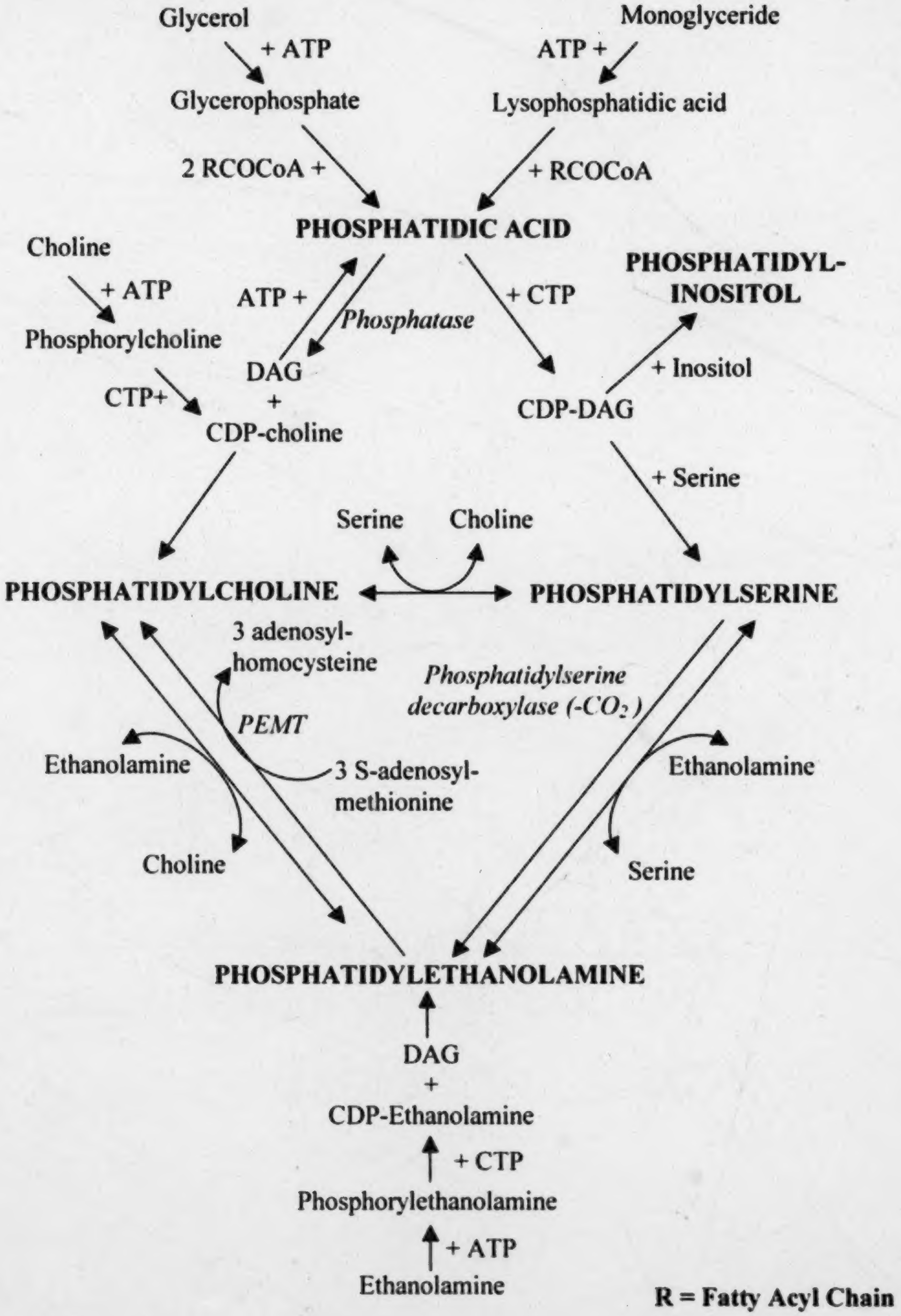


Figure 1.6 Schematic of Phospholipid Synthesis

4. Composition of Phospholipids and Developmental Changes

Past research has examined the changes in whole brain phospholipid composition with development and aging. However, analysis of whole brain does not consider the differences in brain region, cell type, or subcellular fractions as well as the timing of maturation (Sun & Horrocks, 1970). It is well established that the content of each lipid class and their fatty acyl constituents change continuously from fetal life to old age (Rouser & Yamamoto, 1968).

a) Whole Brain

In the human fetus, phospholipid content changes from about 13-17% of the brain dry weight to about 50% in term infants (Svennerholm, 1964). Among the individual phospholipids, PC comprises about half of the phospholipid in fetal brain (Svennerholm, 1964). Rouser & Yamamoto (1968) examined the phospholipids of male human brain from birth to 98 years. It was found that the phospholipid composition changes continuously throughout aging (Rouser & Yamamoto, 1968). Most of the brain phospholipids increase up to age thirty and then start to decline (Rouser & Yamamoto, 1968). Generally, it was found that the greatest increase in brain phospholipids occurred during the first year of life, which was five to fourteen times greater than any other year (Rouser & Yamamoto, 1968).

In rodents, the total brain phospholipids increase markedly from birth to 50 days of age with about a two-fold increase by ten days of age (Cuzner & Davidson, 1968). After ten days, the level of phospholipids in brain starts to plateau (Erickson & Lands, 1959). Wells & Dittmer (1967) examined the morphological changes in rat brain with deposition of lipid classes. Among the phospholipids, the study revealed that during active myelination (10-20 days), there was a rapid increase (2-4 fold) in sphingomyelin (SM) and PA content (Wells & Dittmer, 1967). The other phospholipids, in contrast to SM and PA, showed marked elevations prior to myelination. Therefore, SM and PA may be associated with changes in myelin membrane structures while PC, PE, PS, and PI (about 49-60% of adult brain levels) are associated with non-myelin structures during development (Wells & Dittmer, 1967). In rat brain, when phospholipids are considered as a percentage of the total lipids, there is a fall from 74-56% during maturation (Norton &

Poduslo, 1973). Individual phospholipids expressed relative to total lipid, PE and PS remain constant during development at about 25% and 6%, respectively, whereas PC decreases from 30-18% (Galli & Cecconi, 1967; Norton & Poduslo, 1973). SM increases from 1% to 3-4% by 37 days (Ansell & Spanner, 1961; Marshall et al., 1966).

b) Neuronal and Glial Cells

Many papers have reported the lipid composition of cell types and subcellular fraction in brain, however, only a few have examined the changes in lipid composition during development. Norton & Poduslo (1971) studied the lipid composition of neuronal and glial cell preparation in rats during myelination. The study revealed that neuronal and glial cell lipid composition was similar. Both neuronal and glial cells showed high levels of phospholipid content (71% of total lipid) and neither cell type exhibited obvious changes with age (Norton & Poduslo, 1971). Interestingly, glial cells have less PI but more PS than neuronal cells, and both cell types have less PS than whole brain at any age (Norton & Poduslo, 1971). The levels of SM were similar in the two cell types and did not seem to vary with age, despite observed changes of about 45% in whole brain (Norton & Poduslo, 1971). Thus, the study reveals that both neuronal and glial cell phospholipid composition remain relatively unchanged during development.

c) Synaptic Plasma Membrane

In rat SPM, there is an early rise in total phospholipids, which reflects the general increase in whole brain (Cuzner & Davidson, 1968). Differences in the absolute amount of phospholipids per brain in each fraction were observed (Cuzner & Davidson, 1968). However, the differences observed in amount of phospholipids may be related to the contribution of each fraction to the whole brain weight (Cuzner & Davidson, 1968). For example, myelin and synaptosomes together make up about 50% of the phospholipids in whole brain homogenates while microsomes constitute about 11.5% (Cuzner & Davidson, 1968). Nevertheless, changes in phospholipid content of SPM do occur with age.

Alterations of brain synaptosomal and microsomal membrane content and composition by diet fat was examined by Foot et al. (1982). The composition of these membranes was compared for rats fed different types of fat. Synaptosomal membrane content of PE, ethanolamine plasmalogen, or SM was not affected by dietary fat treatment (Foot et al., 1982). Levels of PC and cholesterol were altered by diet fat (Foot et al., 1982). Increase in PC content of the membrane was strongly correlated with an increase in membrane cholesterol content (Foot et al., 1982).

5. Effect on Sodium-Potassium Adenosine Triphosphatase

Phospholipids are important for optimum activity of the Na, K-ATPase and purified preparation contain 250-300 mol phospholipid/mol enzyme (reviewed by Stahl, 1986). The negatively charged phospholipids, PS and PI are present in these preparations and it has been suggested that they have an important role in modulating enzymatic activity (Kimelberg & Papahadjoupoulos, 1974; Roelofsen, 1981). However, De Pont et al. (1978) exchanged PS for PE in membrane preparations and found that Na, K-ATPase activity was not significantly affected, suggesting that PS is not specifically important for modulating Na, K-ATPase activity. However, this issue has not been completely resolved, since preparations devoid of PS may still contain negatively charged detergents, or tightly bound PI, which might modulate enzymatic activity (Brotherus et al., 1980). Brotherus et al. (1980) suggested that basic amino acids residues of the Na, K-ATPase e.g. arginine and lysine, may be located near the surface boundary of the lipid layers. Specific phospholipids like PS and PI in the bilayer may segregate to match appropriate hydrophobic and hydrophilic portions of the enzyme so that acidic phospholipids are required for optimum Na, K-ATPase activity (Brotherus et al., 1980). Stekhoven et al (1994) reinforced the importance of PS for Na, K-ATPase activity by demonstrating that monoclonal antibodies to PS inhibited the Na, K-ATPase activity. Alternatively, it has been suggested that enzymatic activity is primarily modulated by the fluidity of the acyl chains of the fatty acids present in the bulk membrane phospholipids (Kimelberg & Papahadjoupoulos, 1974). Thus, Na, K-ATPase activity can be modulated by phospholipids, in particular, PS and PI, and/or the fatty acyl constituents of phospholipids. Also, other ATPases, such as, mitochondrial oligomycin-sensitive ATPase, has been shown to be affected by the membrane polar head group content by diet fat, indicated by altered membrane PC to PE ratios with subsequent changes in the thermotropic and kinetic properties of mitochondrial ATPase exchange reactions (Innis & Clandinin, 1981).

E. SPHINGOLIPIDS

1. Sphingomyelin

SM is different from PC, PE, PS, and PI. In SM, a fatty acid is acylated to ceramide instead of glycerol (Ong & Brady, 1973; Figure 1.7). SM is ubiquitously distributed in all membranes of mammalian cells and in serum lipoproteins (Barenholz & Gatt, 1982). Generally, SM distribution coincides with that of cholesterol, and in most cells, there is an increasing gradient of SM and cholesterol compared to other lipids from the nuclear membrane through the various organelles to the plasma membrane (Barenholz & Thompson, 1980). SM is located predominately in the outer leaflet of the plasma membrane (reviewed by Kolesnick, 1991).

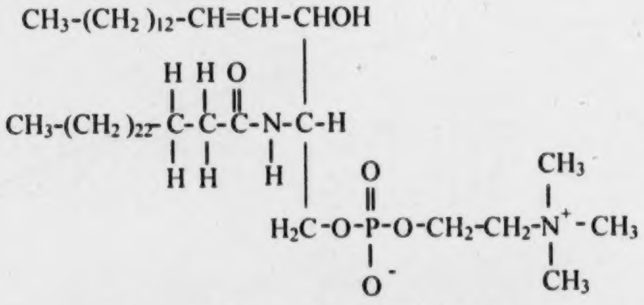


Figure 1.7 Structure of Sphingomyelin

a) Serine Palmitoyl Transferase

The de novo rate-limiting step in SM synthesis begins with condensation of L-serine and palmitoyl-CoA to produce a C18 carbon molecule, D-3-ketosphinganine (Figure 1.8; Merrill & Jones, 1990; van Echten & Sandhoff, 1993). This reaction, catalyzed by serine palmitoyl transferase (SPT) was first demonstrated in cell-free extracts made from the yeast *H. ciferrii* (Stoffel et al., 1967) and later in rat liver (Stoffel et al., 1968) and mouse brain (Braun et al., 1970). SPT requires pyridoxal phosphate for its activity (Brady et al., 1969). This enzyme is highly specific for saturated fatty acyl-

CoA's, particularly, 16-carbon atoms, which explains the prevalence of the 18-carbon sphingoid bases found in most sphingolipids (Karlsson, 1970). SPT has not yet been purified from any organism despite only a 100-fold enrichment in activity (Merrill, 1983). SPT is membrane-bound, particularly, in the endoplasmic reticulum (Mandon et al., 1992). In *S. cerevisiae*, at least two genes, LCB (long chain base) 1 (Buede et al., 1991) and LCB 2 (Nagiec et al., 1994) have been found and are necessary for SPT activity. Mammalian cDNA homologs of both LCB 1 (Hanada et al., 1997; Weiss & Stoffel, 1997) and LCB 2 (Nagiec et al., 1996; Weiss & Stoffel, 1997) have been identified.

b) 3-Ketosphinganine Reductase

3-Ketosphinganine reductase reduces 3-ketosphinganine to dihydrosphingosine (sphinganine; Figure 1.8; Stoffel et al., 1968). This enzyme is NADPH-dependant and is very active in vivo since 3-ketosphinganine is not detected in cells (Merrill et al., 1985; Merrill & Wang, 1986). 3-Ketosphinganine reductase has been shown to be localized to the cytosolic side of the endoplasmic reticulum (Mandon et al., 1992). In *S. cerevisiae* csg 2Δ mutants that were calcium sensitive to temperature, the gene TSC10/YBR265w was found to encode the 3-ketosphinganine reductase (Beeler et al., 1998). However, characterization of 3-ketosphinganine reductase has not been done in mammalian tissues.

c) Sphinganine N-Acyl Transferase

Sphinganine N-acyl transferase (ceramide synthase) is responsible for the acylation of sphingosine with fatty-acyl CoA to form dihydrorceramide (Figure 1.8; Morell & Radin, 1970; Akanuma & Kishimoto, 1979; Merrill & Wang, 1986). However, a fatty acyl CoA independent synthesis of dihydroceramide has also been described (Singh, 1983). The acylation of sphinganine occurs very rapidly in vivo since free sphingosine is not detected in cells unless stimulated by hormones and cytokines (Merrill & Wang, 1986). This reaction has been shown to take place at the cytosolic surface of the endoplasmic reticulum (Mandon et al., 1992; Hirschberg et al., 1993). Shimeno et al. (1998) partially purified sphinganine N-acyl transferase from bovine liver mitochondria. Western blot analysis showed that this enzyme migrated as two major protein bands (subunits) of 62 and 72 kDa (Shimeno et al., 1998). The molecular mass of the enzyme was estimated to be approximately 240-260 kDa (Shimeno et al., 1998). Future studies on the purification of ceramide synthase to homogeneity are needed.

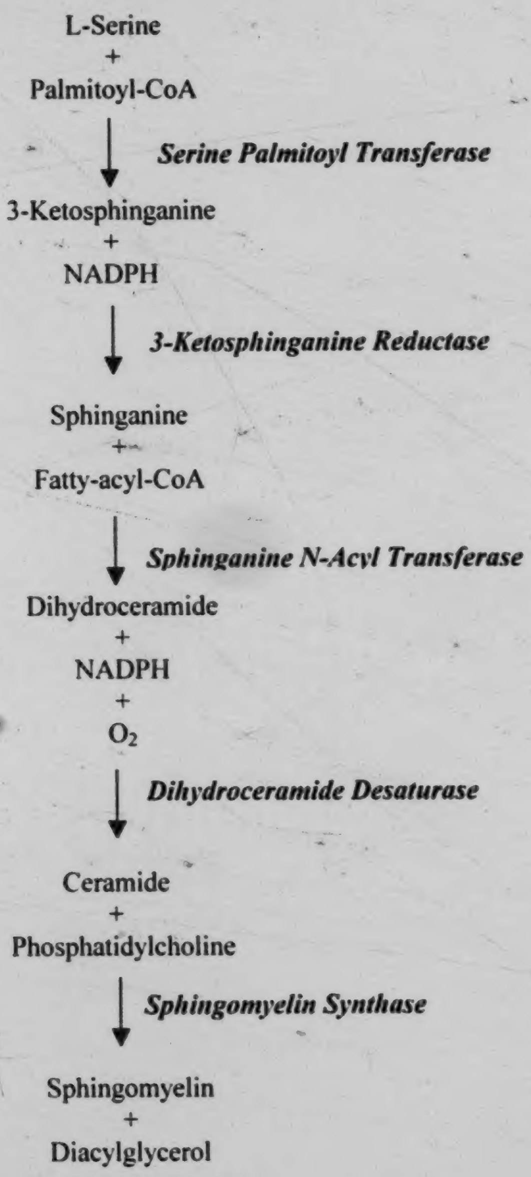


Figure 1.8 Biosynthesis of Sphingomyelin

d) Dihydroceramide Desaturase

Dihydroceramide desaturase catalyzes the conversion of dihydroceramide to ceramide (Figure 1.8; Michel et al., 1997; Geeraert et al., 1997). This reaction introduces a 4,5 trans double bond in the dihydroceramide not sphinganine (Michel et al., 1997; Geeraert et al., 1997; Mikami et al., 1998). Dihydroceramide desaturase in mouse has been found to be localized at the cytosolic face of the endoplasmic reticulum (Mandon et al., 1992). The in vitro assay of this enzyme has recently been described in the microsomal fraction of rat liver (Michel et al., 1998). Recently, Causeret et al (2000) has further characterized dihydroceramide desaturase activity in rat. Dihydroceramide desaturase was found to be enriched in rat liver (Causeret et al., 2000). Subcellular fractionation of liver homogenate and density gradient separation of microsomal fraction demonstrated dihydroceramide desaturase activity to be localized in the endoplasmic reticulum (Causeret et al., 2000). Unfortunately, little is known about this enzyme in humans since it has not been isolated and characterized.

e) Sphingomyelin Synthase

The major pathway for the synthesis of SM is by transfer of the phosphoryl choline groups from PC to ceramide, yielding diacylglycerol and SM (Figure 1.8; Ullman & Radin, 1974). The formation of SM does not occur via CDP-choline (Diringer et al., 1973; Kurtz & Kanfer, 1973; Voelker & Kennedy, 1982). This reaction is catalyzed by SM synthase (PC; ceramide-phosphoryl choline transferase; Ullman & Radin, 1974). SM synthase activity has been detected in isolated plasma membrane and microsomal preparations (Ullman & Radin, 1974). Controversy still exists as to the major site of SM synthesis in vivo. However, it is believed that SM synthesis occurs in the Golgi apparatus using fluorescent analogs of ceramide (Barenholz & Thompson, 1980; Lipsky & Pagano, 1983 and 1985; Merrill & Jones, 1990; Koval & Pagano, 1991). Further evidence for the localization of SM synthesis at the Golgi apparatus was provided by the separation and characterization of subcellular components (Futerman et al., 1990; Jeckal et al., 1990). Interestingly, the brain which is enriched with SM has little SM synthase activity (Nikolova-Karakashian, 1999). Purification and characterization of SM synthase has been difficult because mild detergent treatment causes loss of activity (Nikolova-Karakashian, 2000).

2. Gangliosides

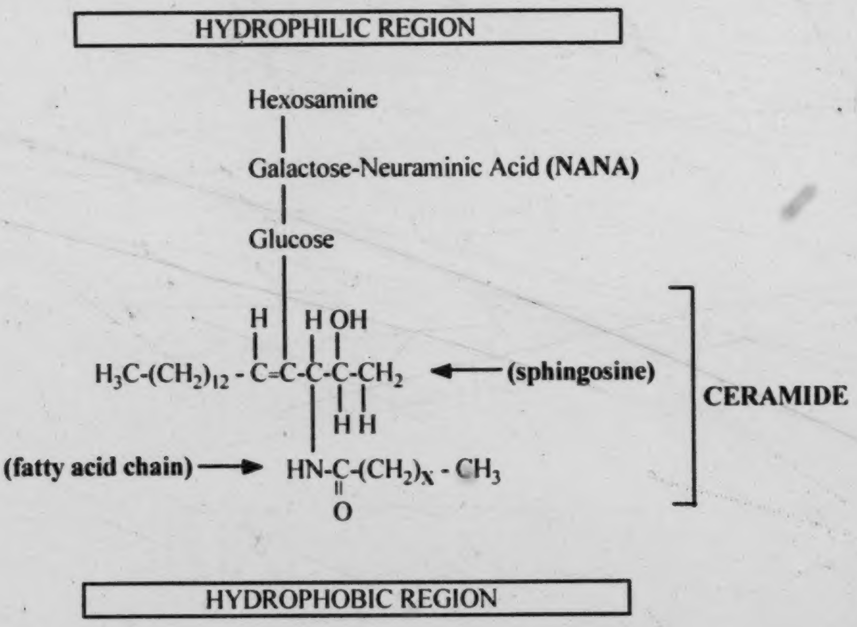
To date, brain lipids, more specifically, gangliosides have not been extensively examined in relation to compositional changes due to alteration in dietary fat. This section will discuss the role of gangliosides and their importance in brain.

a) Nomenclature

Ganglioside nomenclature was developed by Lars Svennerholm in 1963 (Svennerholm, 1963; reviewed by Svennerholm, 1980 and 1988). In Svennerholm's notation, G denotes ganglio-family, the capital letters M, D, T, and Q refer to the number of neuraminic (NANA) or sialic acid residues in the ganglioside (Svennerholm & Raal, 1961; Svennerholm, 1963; reviewed by Brunngraber, 1979). Numbers one, two, or three refer respectively to gangliosides whose total hexose and hexosamine residues are four, three, and two, respectively (reviewed by Brunngraber, 1979 and Svennerholm, 1980 and 1988). Small letters a, b, or c following the numbers distinguish between the position of the sialo-isomers (reviewed by Brunngraber, 1979 and Van Echten & Sandhoff, 1993). This notation developed by Svennerholm used to designate gangliosides is the least complex of all the other systems and will be used to describe the gangliosides in this thesis.

b) Chemical Structure and Composition

Gangliosides, a subclass of glycosphingolipids, are acidic complex lipids that are composed of two regions (Figure 1.9; reviewed by Rapport, 1981 and Svennerholm, 1988). The first is the hydrophobic region. The hydrophobic region consists of two long chain molecules: a long-chain base, sphingosine, which can be eighteen or twenty carbons in chain length, and a fatty acid chain (usually stearic acid; C18:0) that is linked to the sphingosine by an amide bond (reviewed by Rapport, 1981 and Svennerholm, 1988). The two long chain molecules together are called a ceramide (reviewed by Rapport, 1981 and Svennerholm, 1988). The second is the hydrophilic region, which is composed of sugar residues; mainly glucose, galactose, and N-acetylgalactosamine (hexosamine) that are bonded to the primary hydroxyl group of the sphingosine (reviewed by Brunngraber, 1979). In addition to the sugar residues in the hydrophilic region, there are neuraminic acid residues, which characterize gangliosides from other glycosphingolipids (Klenk, 1942; reviewed by Rapport, 1981 and Svennerholm, 1988). These neuraminic acid residues can attach to galactose or other neuraminic acid molecules by a glycoside bond (reviewed by Rapport, 1981 and Svennerholm, 1988). Thus, this addition of neuraminic acid in the hydrophilic region of the molecule gives the gangliosides their acidic nature and different mobility in thin-layer chromatography.



x = varying number of carbon atoms

Figure 1.9 Structure of a Ganglioside

There are more than sixty different ganglioside molecules that have been identified all varying in structure and composition (reviewed by Rapport, 1981 and Svennerholm, 1988). Variations in these structures result from a number of factors, such as, the number of sugar residues, the number of neuraminic acid residues, the position of attachment of the neuraminic acid, the presence of fucose residues, the substitution of glucosamine for galactosamine, and the presence of O-acetyl groups (reviewed by Rapport, 1981 and Svennerholm, 1988). However, this thesis will focus only on four of these gangliosides: GM1a, GD1a, GD1b, and GT1b (Figure 1.10).

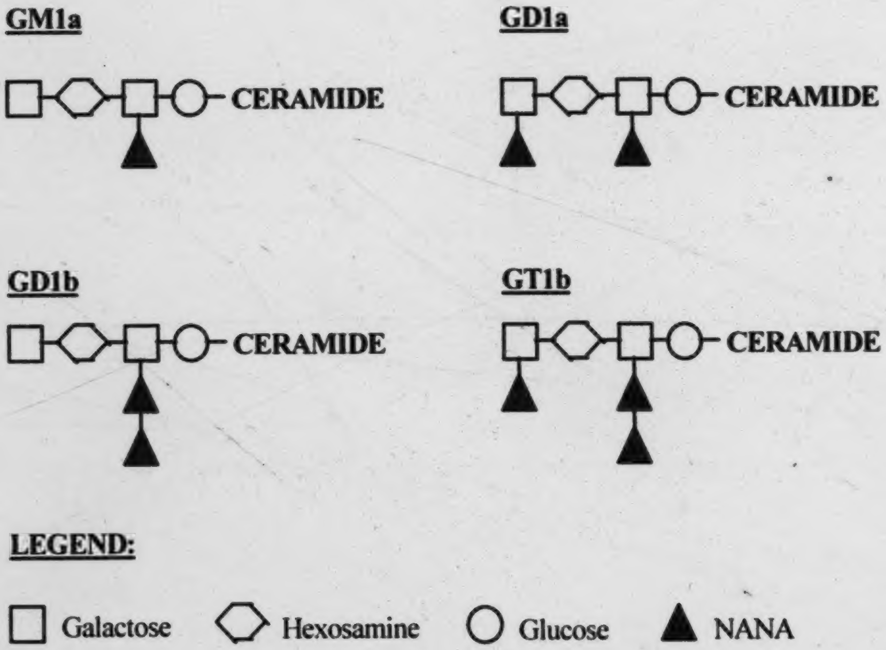


Figure 1.10 Gangliosides GM1a, GD1a, GD1b, and GT1b

c) Cellular Localization

Gangliosides occur on the outside of cellular plasma membrane especially at the synaptic regions in which there are enriched amounts (reviewed by Wiegandt, 1985 and Svennerholm, 1988; Figure 1.11). However, it is not clear whether or not gangliosides are evenly or unevenly distributed on the outer surface of the neuron (reviewed by Wiegandt, 1985 and Svennerholm, 1988). Low levels of gangliosides have also been detected in the membrane of the Golgi apparatus, endoplasmic reticulum, and lysosome (reviewed by Wiegandt, 1985). These findings suggest that ganglioside metabolism is localized in these structures and that they may serve some special biological functions (reviewed by Wiegandt, 1985 and Svennerholm, 1988).

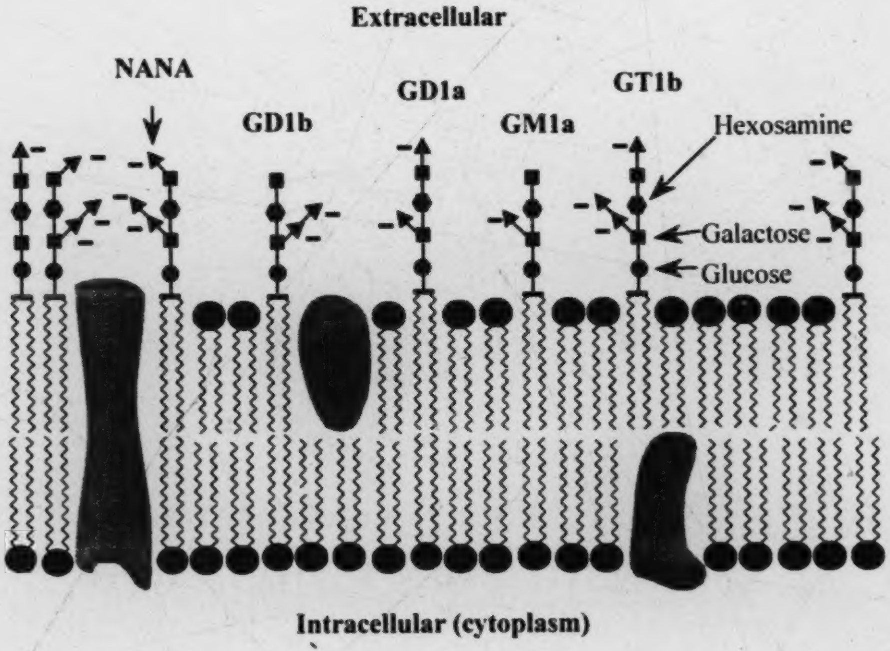


Figure 1.11 Gangliosides in Cell Membrane

d) Distribution in Central and Peripheral Nervous System

Gangliosides are found in most, if not all, mammalian tissues with their concentrations being highest in gray matter of the brain (reviewed by Rapport, 1981 and Wiegandt, 1985). Sixty-five to eighty-five percent of the gangliosides content in mammalian brain is of four molecular species: GM1a, GD1a, GD1b, and GT1b (reviewed by Rapport, 1981; Wiegandt, 1985). However, gangliosides from the peripheral nervous system are quite different to those of the central nervous system (reviewed by Wiegandt, 1985). Peripheral nervous system gangliosides are of the lactoseries rather the ganglio-series found in the central nervous system (reviewed by Wiegandt, 1985). This difference in composition might be related to their function. Nevertheless, gangliosides localized in the central nervous system are different in sugar composition than in peripheral nervous system.

e) Composition in Neuronal Membranes

Gangliosides are the most specific lipids in neuronal membranes. The amount of neuraminic acid per gram wet weight of human brain gray and white matter are approximately 880 and 275 µg, respectively (reviewed by Thomas & Brewer, 1990). Although gangliosides constitute approximately 10% of the total membrane-bound lipids (Wiegandt, 1985) on the whole neuron surface, gangliosides are more concentrated in the synaptic region (Hansson et al., 1977). In the SPM, there are higher ratios of ganglioside to phospholipid compared to the plasma membrane of most non-neuronal tissues (reviewed by Thomas & Brewer, 1990). The SPMs from human and rat brain contains approximately 15% GM1a, 40% GD1a, 14% GD1b, and 21% GT1b (reviewed by Thomas & Brewer, 1990). Interestingly, it has been shown that there is an increased amount of GD1b and GT1b (70-80%) in the synaptic junction (reviewed by Thomas & Brewer, 1990).

f) Physiochemical Properties

Gangliosides, because of their hydrophobic (ceramide) and hydrophilic (oligosaccharide) regions, are amphiphilic and can form micelles above 10^{-10} M range (Corti et al., 1987). In contrast to other amphiphilic molecules like phospholipids, gangliosides do not have bilayer structures (reviewed by Curatolo, 1987). In addition, the neuraminic acid residues on the gangliosides molecule contribute significantly to the negative electrical charge on the membrane (reviewed by Langner et al., 1988 and Thompson & Brown, 1988).

It is well established that under certain conditions gangliosides can form microdomains (clusters) (Myers et al., 1984; Masserini & Freire, 1986). These clusters indicate that not only do gangliosides have chemical diversity but also aggregational diversity which may play a significant role in allowing ganglioside-ligand interaction to occur (Myers et al., 1984; Masserini & Freire, 1986).

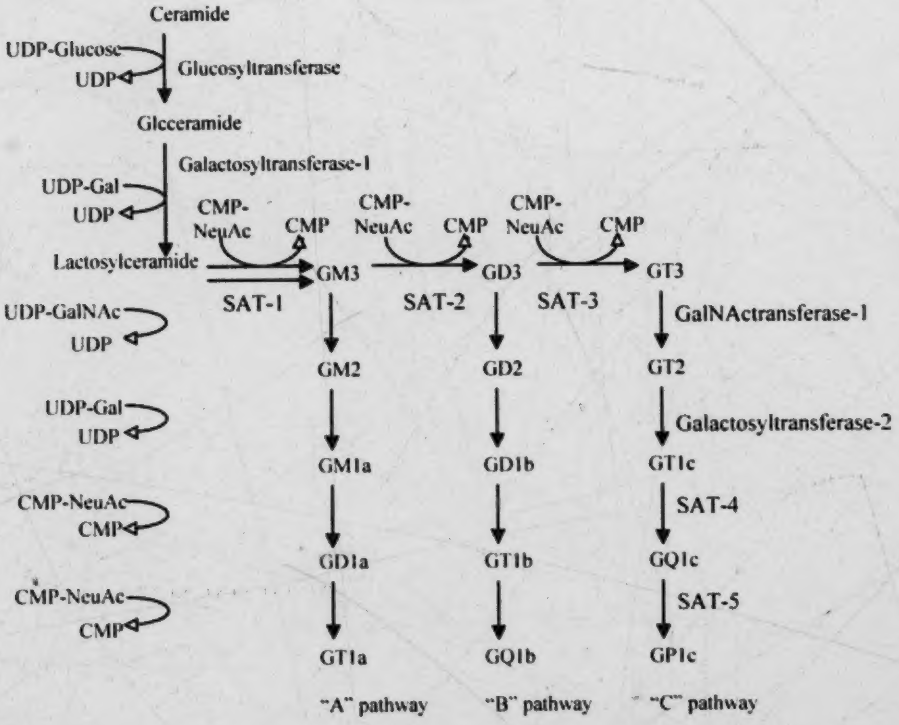
Due to the location of the gangliosides on the outer leaflet of the synaptic membrane, it is not surprising that gangliosides possess high binding potential. Numerous agents such as viruses, serotonin, fibronectin, and antibodies have been demonstrated to bind primarily the oligosaccharide region of the ganglioside (Tettamanti & Masserini, 1987). The specificity of binding of these agents seems to be due to a particular sequence of oligosaccharides that can be shared by different gangliosides. This is most evident with cholera toxin binding GM1a (Holmgren et al., 1994) and Fuc-GM1a (Masserini et al., 1992).

Gangliosides interaction with proteins in the membrane have also been demonstrated, but it is not clear whether aggregated or molecularly dispersed forms are involved (Myers et al., 1984). These ganglioside-protein interactions have been seen specifically in GM1a and GT1b and it is speculated that these proteins are responsible for ganglioside internalization via endocytosis (Yasuda et al., 1988; Tiemeyer et al., 1990; Fueshko & Schengrund, 1990; Sonnino et al., 1992).

g) **Metabolism**

i) Biosynthesis

The oligossacharide chain of gangliosides is formed by a stepwise addition of nucleotide bound monosaccharides like uridine diphosphate (UDP)-galactose or cytidine monophosphate (CMP)-neuraminic acid onto an acceptor molecule, ceramide, which is the product of condensation and reduction between L-serine and palmitoyl-CoA in the endoplasmic reticulum (Walter et al., 1983). The transfer of nucleotide-activated monosaccharides takes place in the Golgi apparatus, with the involvement of specific membrane-bound glycosyltransferases (Hirschberg & Snider, 1987). The reaction sequences for the biosynthesis of gangliosides and the involved enzymes are shown in Figure 1.12. Initiation of the three distinct ganglioside pathways (A, B, C) are dependant on the strict specificity of sialic acid transferases (SAT), SAT-1, SAT-2, and SAT-3, which act on lactosylceramide, GM3, and GD3, respectively (reviewed by Tettamanti & Masserini, 1987). Further glycosylation are catalyzed by enzymes like N-acetylgalactosamine (GalNAc) transferase-1, which converts GM3 to GM2, GD3 to GD2, and GT3 to GT2 (reviewed by Tettamanti & Masserini, 1987). Similarly, (1) galactosyltransferase-2 catalyzes the transformation of GM2 to GM1a, GD2 to GD1b, and GT2 to GT1c; (2) SAT-4 converts GM1a to GD1a, GD1b to GT1b, and GT1c to GQ1c; and (3) SAT-5 converts GD1a to GT1a, GT1b to GQ1b, and GQ1c to GP1c (reviewed by Tettamanti & Masserini, 1987). After synthesis in the Golgi apparatus of the neuronal cell body, the gangliosides travel by fast axonal transport to the SPMs (reviewed by Ledeen, 1989).



UDP = Uridine diphosphate Gal = Galactose Glc=glucose GalNAc = N-acetylgalactosamine

CMP = Cytidine monophosphate NeuAc = Neuraminic acid SAT= Sialic acid Transferase

Figure 1.12 Biosynthesis of Gangliosides

The regulation of ganglioside biosynthesis has not been fully elucidated. Changes in the pattern of ganglioside expressed on cells during development, differentiation, and oncogenic transformation have been observed, as well, as differences in the composition of mammalian organs (reviewed by Ledeen, 1989, Hakomori, 1990 and Rahmann, 1992). The changes or differences observed in ganglioside patterns are thought to be regulated at the transcription level via the expression of certain glycosyltransferase (Sandhoff & van Echten, 1993). Using transfected cells with cDNA probes for specific glycosyltransferase has been shown to increase the respective ganglioside (Kojima et al., 1994). Other studies have suggested that glycosyltransferase might be regulated by protein phosphorylation/dephosphorylation reaction (Scheidler & Dawson, 1986). Sugar nucleotides can also modulate the synthesis of gangliosides (Burczak et al., 1984). The sugar nucleotide is transported into the Golgi by specific transport proteins in the Golgi membrane, which may regulate the availability of sugar nucleotides for ganglioside biosynthesis (Burczak et al., 1984).

ii) Biodegradation

Ganglioside catabolism consists of the sequential removal of individual sugar residues by exoglycohydrolases, with formation of ceramide (Sandhoff et al., 1987). Ceramide is then degraded by ceramidase (CDase) into sphingosine and fatty acid (Spence et al., 1986). The glycohydrolases involved in neural ganglioside degradation reside in the lysosomes (Riboni et al., 1991). In fact, it was shown by Fiorilli et al. (1989) that in highly purified lysosome preparations from rat brain that glycohydrolases affecting ganglioside catabolism were present. However, if any of these enzymes in the lysosomes are absent or defective, gangliosidosis develops (Fiorilli et al., 1989). Therefore, demonstrating the significance of these enzymes in ganglioside catabolism.

(1) Glycosidases

The constitutive degradation of gangliosides occurs in the acidic compartments of the cell: endosomes and lysosomes. Gangliosides reach the lysosomal compartment by endoscytic membrane flow, presumably on the surface of intraendosomal and intralysosomal vesicles (Sandhoff et al., 1998). In the lysosomes, the gangliosides are cleaved by stepwise action of hydrolytic enzymes (Sandhoff et al., 1998). Glycosidases cleave off the sugar residues from the non-reducing end of gangliosides (Sandhoff et al., 1998). Gangliosides with short oligosaccharide chains of less than four sugar residues, the glycosidases need protein cofactors, sphingolipid activator protein (SAP; Sandhoff et al., 1998). There are four SAP that are involved in ganglioside degradation: SAP-A, SAP-B, SAP-C, and SAP-D (Sandhoff et al., 1998). These proteins seem to facilitate interaction of gangliosides with glycosidases in or at the membrane (Kolter & Sandhoff, 1998). Deficiencies of glycosidases and/or SAPs cause accumulation of sphingolipids in lysosomes leading to lysosomal storage diseases such as Tay-Sachs and Gaucher's disease (Kolter & Sandhoff, 1998).

(2) Ceramidases

CDase hydrolyses ceramide to a free sphingoid base (usually sphingosine) and a fatty acid (Gatt, 1966). CDase activity was first characterized as an enzyme with an acidic pH optimum present in all tissues examined (Gatt, 1966; Yavin & Gatt, 1969). A genetic deficiency of this enzyme was later shown to account for Farber's disease where there is an accumulation of ceramides in the lysosomes of spleen, cerebellum, fibroblasts, and kidney (Levade et al., 1995; Nikolova-Karakashian & Merrill, 2000). CDases with neutral (pH 7.6) and alkaline (pH 9.0) pH optimum have been found (Nilsson et al., 1969; Morell & Braun, 1972; Yada et al., 1995). Recently, the acid CDase from human urine was purified (Bernardo et al., 1995) and the cDNA encoding human and mouse acid CDase has been cloned (Koch et al., 1996; Li et al., 1998).

Neutral CDase was purified and characterized in mouse liver (Tani et al., 2000). The enzyme was a monomeric polypeptide with a molecular mass of 94 kDa and was highly glycosylated with N-glycans (Tani et al., 2000). The amino acid sequence of the mouse liver neutral CDase was homologous to that of the alkaline CDase in *P. aeuriginosa* (Okino et al., 1998) and *D. discoideum* (Tani et al., 2000). The neutral CDase was cloned from mouse liver and the cDNA encoded for polypeptide of 756 amino acids with nine putative N-glycosylation sites (Tani et al., 2000). Northern blot analysis revealed that the enzyme is expressed widely in mouse tissues with liver and kidney showing the highest levels (Tani et al., 2000).

Two alkaline CDase have been best characterized in guinea pig skin epidermis, one to apparent homogeneity and the other only partially (Yada et al., 1995). These two enzymes were membrane-bound, and their estimated molecular masses on SDS-PAGE were 60 and 148 kDa, respectively. Recently, El Bawab et al. (1999) purified a membrane-bound non-lysosomal (neutral/alkaline) CDase from rat brain and human mitochondria (El Bawab et al., 2000) to apparent homogeneity. The enzyme appeared as a single protein of 90 kDa (El Bawab et al., 1999). Interestingly, the activity of this enzyme was stimulated by PS and PA and does not require cations (El Bawab et al., 1999).

(3) Sphingosine Kinase

Sphingosine kinase catalyzes the phosphorylation of long-chain sphingoid bases (usually sphingosine) on their primary hydroxyl group to sphingosine-1-phosphate (SPP; Kohama et al., 1998). Rat sphingosine kinase has been purified to homogeneity with an apparent molecular mass of approximately 49 kDa (Kohama et al., 1998). Based on peptide sequences derived from the purified rat sphingosine kinase, the mammalian sphingosine kinase were cloned from the mouse (Kohama et al., 1988). Amino acid sequence analysis indicate that sphingosine kinases are correlated to other known kinases, yet comparison of sphingosine kinase sequence with that cloned from *S. cerevisiae* (Dickson et al., 1997) and *C. elegans* (Kohama et al., 1998) reveals several domains that are highly conserved in all of these sphingosine kinases (Kohama et al., 1998). Northern blot analysis of mouse tissues showed that sphingosine kinase mRNA was most abundant in the adult lung and spleen (Kohama et al., 1998). Further characterization of mammalian sphingosine kinase in mammalian tissues are needed.

(4) Sphingosine-1-Phosphate Lyase

Sphingosine-1-phosphate lyase belongs to the class of pyridoxal phosphate dependant carbon-carbon lyases and acts on the 1-phosphorylated derivatives of sphingoid base (sphingosine; Merrill & Wang, 1992; Zhou & Saba, 1998). The cleavage products are an aliphatic fatty aldehyde (2-trans-hexadecanal) and phosphoethanolamine (Merrill & Wang, 1992; Zhou & Saba, 1998). This enzyme is present in all tissues in vertebrates, invertebrates, plants, fungi, and unicellular protozoa (Merrill & Wang, 1992; Zhou & Saba, 1998). The sphingosine-1-phosphate lyase is associated with the endoplasmic reticulum and has its catalytic site facing the cytosol (Merrill & Wang, 1992; Zhou & Saba, 1998). The yeast sphingosine-1-phosphate lyase cDNA has been cloned (Saba et al., 1997) and subsequently by homology the murine (Zhou & Saba, 1998). The mRNA encodes a protein with a molecular mass of approximately 61-65 kDa (Zhou & Saba, 1998). Characterization of a human sphingosine-1-phosphate lyase has not been done.

iii) Recycling

Studies administrating exogenous radioactive gangliosides to cultured cells or to animals to ascertain recycling processes in ganglioside biosynthesis have been very useful. It was demonstrated in liver (Ghidoni et al., 1983; Trinchera & Ghidoni, 1990) and in cultured cerebellar granule cells (Riboni & Tettamanti, 1991) that gangliosides upon degradation release galactose, N-acetylgalactosamine, neuraminic acid, fatty acid, and sphingosine that are re-used for the synthesis of new gangliosides, glycoproteins, phospholipids, and sphingomylein. Moreover, the recycling process appeared to be blocked by inhibiting lysosome function by chloroquine or preventing endocytosis (Riboni & Tettamanti, 1991; Riboni et al., 1992). Thus, suggesting that lysosomes play a role in ganglioside recycling processes.

h) Composition and Developmental Changes in Brain

Early research has demonstrated that there is an increase in concentration for all major gangliosides with age in the brain of rats and humans, but the rate of increase of each ganglioside is different (Svennerholm, 1964; Suzuki, 1965). Vanier et al. (1971) proposed that there are three major periods of ganglioside development. The first period is characterized by a growth of neurons and glial cells with a moderate increase in ganglioside concentration (Vanier et al., 1971). In this period, GM1a and GT1b predominate with GD1a gradually increasing in concentration (Vanier et al., 1971). The duration of this period is until birth for rats and until the twenty-fifth week of gestation in humans (Vanier et al., 1971). The second period shows the outgrowth of dendrites and axons with the production of neural connection (Vanier et al., 1971). This period corresponds to the first ten days after birth in rats and the thirtieth week of gestation to term in humans (Vanier et al., 1971). In this period, there is a maximal rate of increase in ganglioside concentration for GD1a but a decline for GM1a and GT1b (Vanier et al., 1971). The last period which represents between ten and twenty days of age in the rat and the first eight months in humans demonstrates an increase in neural connection as well as the beginning of myelination (Vanier et al., 1971). During this stage, GD1a becomes the predominant ganglioside fraction (Vanier et al., 1971).

Changes in the ceramide composition with increasing age have also been shown in human brain gangliosides (Mansson et al., 1978). Until the age of ten, the ratio of twenty carbon and eighteen-carbon sphingosine increases rapidly (Mansson et al., 1978). The sphingosine content in brain levels off with 60-70% of sphingosine being the twenty-carbon type after thirty years of age (Mansson et al., 1978). The fatty acids composition of ceramide from gangliosides also changes with age (Mansson et al., 1978). At birth, 93% of the ganglioside fatty acids is C18:0, whereas at age 98 only 78% of this fatty acid is present (Mansson et al., 1978). Concomitantly, there is an increase from 3 to 9% of fatty acids in gangliosides with twenty or more carbons (Mansson et al., 1978). Thus the developmental patterns of individual gangliosides in the brain are different, however, between rat and human brain the patterns are quite similar (reviewed by Ledeen, 1985 and Yu and Saito, 1989). Hence, the rat serves as a good model for extrapolating to humans.

i) Biological Functions

Presently, the function of gangliosides have not been fully elucidated (Tettamanti & Riboni, 1994). It appears that gangliosides can serve multivalent roles that are now beginning to be understood. There is increasing evidence that gangliosides can serve as: (1) molecular tools for appropriate interactions between the cell plasma membrane an extracellular substances (Bird & Kimber, 1984), (2) modulators of membrane-bound or intracellular functional proteins like receptors, ion channels, enzymes, and ion carriers (Benos & Sorscher, 1992), and (3) precursors of intracellular metabolic regulators, for examples, sphingosine and ceramide derivatives (Hannun & Bell, 1989; Merrill & Jones, 1990;Younes et al., 1992)

j) Sphingolipid Metabolites as Modulators of Cellular Processes

i) Sphingomyelinase Cycle

The SM cycle was first described by Okazaki et al (1989 and 1990). This cycle is composed of six major components (1) a ligand like a hormone or a cytokine which can bind to a receptor on the surface of a cell ; (2) a receptor on the surface of the cell which can receive a ligand; (3) a SM which is predominantly at the cell's plasma membrane that can act as the substrate for SMase; (4) the SMase which hydrolyzes the SM; (5) the ceramide and the choline-phosphate that results from the SM hydrolysis; and (6) enzymatic pathways for the resynthesis of SM. This cycle is initiated when an agonist such as TNF- alpha (Kim et al., 1991; Dressler et al., 1992) or IL-1 (Mathias et al., 1993) binds to a receptor on the cell this causes the activation of SMase. The ceramides generated by SMase action can now act as a second messenger by transmitting messages through the cell's interior like inhibiting or stimulating other enzymes within the cell like phospholipase Ð (PLD) and ceramide-activated protein kinase (CAPK), respectively (Mathias et al., 1991; Hannun & Linardic, 1993). Consequently, the inhibition or stimulation of these intracellular enzymes by ceramides can alter biological response by affecting other downstream effectors or substrates. The SM cycle is completed when SM and ceramide return to basal levels.

ii) Ceramide

(1) Role in Growth Suppression

Growth suppression is the inability of a cell to grow when the proper factors e.g. hormones or cytokines are received by the cell. Experiments with HL-60 cell line revealed that these cells which responded to 1-alpha -25-dihydroxyvitamin D_3 to differentiation caused the inhibition of their cell growth (Miyaura et al., 1981). With the recent knowledge that 1-alpha -25-dihydroxyvitamin D_3 caused SM hydrolysis in these cells, it was suggested that the growth inhibition effects of 1-alpha -25-dihydroxyvitamin D_3 might be mediated via the SM cycle (Hannun & Linardic, 1993). The findings by

Wolff et al. (1994) confirmed this idea by demonstrating that cell permeable C2-ceramide can cause growth inhibition by down regulation of c-myc proto-oncogene, which is known to be up-regulated in cells during proliferation. This down regulation of c-myc which occurs within thirty minutes after receiving C2-ceramide is due to the inhibition of RNA accumulation by blocking transcription elongation between the first and third exon in the gene (Kronke et al., 1987; Tobler et al., 1987). This mechanism of ceramide on c-myc is similar to the effects of TNF-alpha (Wolff et al., 1994). Hence, the connection between C2-ceramide inducing the down regulation of c-myc by similar mechanism is important because it shows a link between extracellular messengers, signal transduction and nuclear events.

(2) Role in Cell-Cycle Arrest

Initial studies using the HL-60 cells demonstrated anti-proliferative activity of ceramides (Okazaki et al., 1990). In subsequent studies with serum-dependant MOLT-4 leukemia cells, the withdrawal of serum led to a significant accumulation of ceramides this coincided with the development of cell-cycle arrest in G_0G_1 phase (Jayadev et al., 1995). Further investigation with this cell line with C6-ceramides revealed a specific arrest in the cell cycle that was dose and time dependant (Jayadev et al., 1995). The specificity of the cell cycle arrest by C6-ceramide was demonstrated when C6-dihydroceramide did not result in cell cycle arrest. The retinoblastoma gene product (Rb), a tumor suppressor or nuclear phosphoprotein has been implicated as an important inhibitor of cell cycle progression (Weinberg, 1990). In the serum-dependant Molt-4 cell line the removal of serum resulted in a substantial amount of dephosphorylation of Rb (Weinberg, 1990). The dephosphorylation of Rb is the mechanism that is responsible for the cell cycle arrest (Weinberg, 1990). It has been shown *in vitro* that 10- 20 uM of C6-ceramide can cause an early dose-dependant dephosphorylation of Rb which leads to cell cycle arrest within four hours (Dbaibo et al., 1995). Therefore, linking intracellular ceramide generation to Rb dephosphorylation, which results in cell cycle arrest.

(3) Role in Cell Differentiation

In HL-60 cell line there appears to be a direct relationship between activation of SM signaling pathway and the differentiation of HL-60 cells (Kim et al., 1991; Okazaki et al., 1989 and 1990). When 1-alpha -25-dihydroxyvitamin D_3 were added to these cells in tissue culture the HL-60 cells began to differentiate along the monocytic lineage (Okazaki et al., 1989 and 1990). Further, the addition of C2-ceramide (a short-chain fatty acid ceramide) and 1-alpha -25-dihydroxyvitamin D_3 at sub-threshold concentrations also induced monocytic differentiation in HL-60 cells (Okazaki et al., 1989 and 1990). In addition, other studies have shown that TNF-alpha and IFN-gamma were also able to cause differentiation of the HL-60 cells to monocytes (Kim et al., 1991). The effects seen in these cell lines by 1-alpha -25-dihydroxyvitamin D_3, TNF-alpha, and IFN are due to these agonists binding onto a cell surface receptor that causes the activation of SMases which in turn can cleave SM to ceramide and choline-phosphate (Kim et al., 1991; Dressler et al., 1992). The ceramide, in turn, by some unknown mechanism causes the activation of protein kinase C (Kim et al., 1991). The activated protein kinase C can then cause phosphorylation of an unknown substrate which can cause the transcription factor (NF-kB) to bind to a specific region on a DNA in the nucleus allow the HL-60 cells to differentiate into the observed monocytes (Kim et al., 1991).

(4) Role in Apoptosis

Apoptosis or programmed cell death is an integral component of embryonic development and organogenesis and is characterized by distinct morphological features like nuclear and cytoplasm shrinking and fragmentation, membrane blebbing and breakdown of DNA by specific endonucleases as seen on a agarose gel electrophoresis of DNA ladder indicative of DNA degradation (Ribeiro, 1993). Studies with C2- ceramide on U937 myeloid cells resulted in potent induction of internucleosomal DNA fragmentation which were seen as early as two hours after 1- 6 uM of C2-ceramide was added (Hannun & Bell, 1993; Merrill et al., 1997). However, the dihydroceramide (a ceramide with no 4-5 trans double bond) when given to these same cells did not demonstrate apoptosis (Hannun & Bell, 1993; Merrill et al., 1997). The target for the ceramide-induced apoptosis was shown in vitro to be a ceramide-activated protein phosphatase (CAPP; Hannun & Obeid, 1995; Merrill et al., 1997). CAPP is a serine / threonine phosphatase of the PP2A subfamily. The PP2A subfamily is defined by a common catalytic subunit (C), which may exist as a monomer, as a heterodimer with an A-subunit of unknown function or as a heterotrimer with the A and an additional B-subunit (Hannun & Obeid, 1995; Merrill et al., 1997). The B-subunit appears to suppress the activity of the phosphatases and has been suggested to function as a regulation of cellular phosphatase activity (Hannun & Obeid, 1995; Merrill et al., 1997). Ceramides activate the heterotrimer phosphatase but not monomeric or heterodimeric PP2A, thus demonstrating a requirement for the B-subunit of PP2A in mediating the activation by ceramide (Hannun & Obeid, 1995; Merrill et al., 1997). Therefore, the mechanism by which ceramides can induced apoptosis in these cells could be that the agonists such as TNF-alpha, fas-ligand, and ionizing irradiation (Uckun et al. 1992 and 1993) can cause activation of SMases which generates ceramides that cause the activation of CAPP and its down regulation of c-myc which in turn can lead to apoptosis (Cifone et al., 1994). Further research has also shown that Jurkat T cells and HUT-78 (a human T-cell lymphoma line) cells are induced to apoptosis by ceramides.

iii) Sphingosine

Sphingosine-1-phosphate (SPP) produced by sphingosine kinase has been reported to be involved in the modulation of cell functions. Cellular processes modulated by SPP include proliferation (Zhang et al., 1991; Olivera & Spiegel, 1993; Rani et al., 1997), survival (Cuvillier et al., 1996; Kleuser et al., 1998), organization of the cytoskeleton (Bornfeldt et al., 1995; Wang et al., 1997), motility (Wang et al., 1999), neurite retraction and cell rounding (van Brocklyn et al., 1999), and differentiation (Edsall et al., 1997; Rius et al., 1997). SPP has also been shown to modulate several signaling pathways, including the activation of phospholipase D leading to the formation of PA (Desai et al., 1992); activation of the Raf/MEK/ERK signaling cascade (Wu et al., 1995), and mobilization of calcium from internal stores via a mechanism that is independent of PI hydrolysis and C20:4n-6 release (Mattie et al., 1994; Melendez et al., 1998). Moreover, SPP can have dual actions, acting intracellularly as a second messenger and receptors (van Brocklyn et al., 1998).

F. CHOLESTEROL

Extensive studies on the biosynthesis of cholesterol in the central nervous system have been performed (Galli et al., 1968; Gautheron et al., 1969; Fumagalli et al., 1969). The endoplasmic reticulum enzyme HMG-CoA reductase catalyzes the rate-determining step in the synthesis of cholesterol and other polyisoprenoid compounds in animal cells (reviewed by Goldstein & Brown, 1990). A previous study has shown that the rate of brain cholesterol biosynthesis is high in the fetus and newborn animal and that as the animal matures, cholesterol synthesis in brain is low (Dietschy, 1997).

Cholesterol (Figure 1.13) and cholesterol esters are abundant in mammalian tissues but can also be found in lesser quantity in mitochondria, Golgi complexes, and nuclear membranes (reviewed by Yeagle, 1985). Plasma membranes are highly enriched in cholesterol. In SPM, cholesterol accounts for over 40 mol % of the total membrane lipid (Wood et al., 1989; Schroeder et al., 1991). Cholesterol in the SPM is not evenly distributed throughout but is located in different pools or domains (Schroeder et al., 1991; Wood et al., 1993). Lateral domains of cholesterol have been described and these domains have been identified in neuronal tissue (Leibel et al., 1987; Rao et al., 1993; Wood et al., 1993). In addition to lateral domains, transbilayer cholesterol domains (exofacial and cytofacial leaflet) have also been described in neuronal tissues (Wood et al., 1990; Igbavboa et al., 1996 & 1997). The SPM cytofacial leaflet contains over 85% of the total SPM cholesterol (Igbavboa et al., 1996 & 1997).

Cholesterol interrelates among the phospholipids of the membrane, with its hydroxyl group at the aqueous interface and the remainder of the molecule within the bilayer (reviewed by Kabara, 1973). The importance of cholesterol in mammalian cells is most likely for regulation and maintenance of membrane properties such as, membrane fluidity and rigidity (reviewed by Yeagle, 1991) which in turn may modulate the activities of numerous membrane proteins (reviewed by Bloch, 1965). The interaction of cholesterol with membrane proteins has been described in both neuronal and non-neuronal tissues (Michelangeli et al., 1990; reviewed by Bastiaanse et al., 1997). However, the biological role of cholesterol in neuronal cells is still not completely understood (Yao et al., 1988; Yao, 1988).

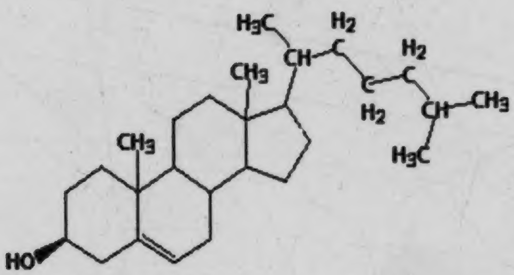


Figure 1.13 Structure of Cholesterol

G. N-6 AND N-3 FATTY ACIDS

1. Classification

N-6 and n-3 fatty acids are two families of PUFAs that can be distinguished from each other by the position of their first double bond (reviewed by Gurr & Harwood, 1991). C18:2n-6 and C18:3n-3 are examples of n-6 and n-3 fatty acids which have their first double bond six and three carbons away from the terminal methyl carbon, respectively (Figure 1.14; reviewed by Gurr & Harwood, 1991). These two fatty acids, in addition to being the precursors of C20:4n-6 and C22:6n-3 biosynthesis (Figure 1.14) are essential for mammals (reviewed by Gurr & Harwood, 1991 and Sprecher, 2000). These fatty acids are essential because mammals do not have the enzymes necessary for inserting double bonds beyond the ninth carbon atom in the fatty acid chain (reviewed by Gurr & Harwood, 1991). Therefore, these fatty acids must be supplied by the diet (Sprecher, 2000).

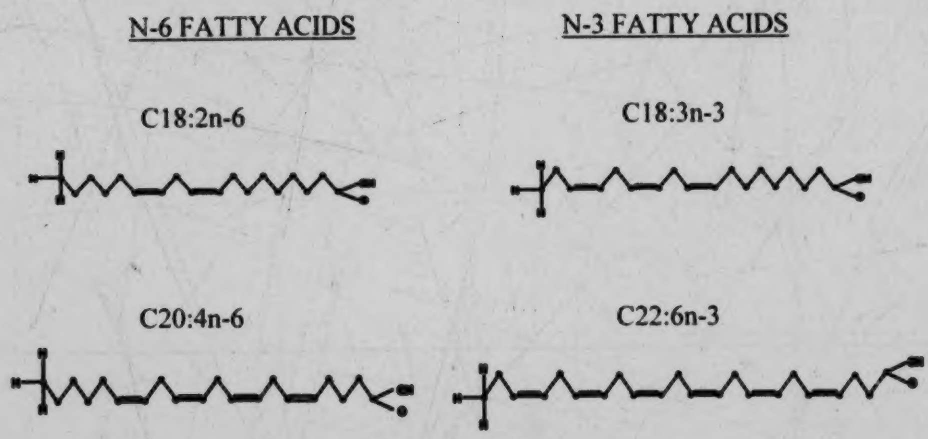


Figure 1.14 N-6 and N-3 Fatty Acid Structures

2. Metabolism

The metabolism of n-6 and n-3 fatty acid involves several enzymes called desaturases and elongases (reviewed by Gurr & Harwood, 1991 and Sprecher, 2000; Figure 1.15). The formation of long-chain PUFAs from C18:2n-6 and C18:3n-3 begins with the delta-6 desaturase and is followed by elongation to a twenty-carbon molecule (reviewed by Gurr & Harwood, 1991 and Sprecher, 2000; Figure 1.14). This twenty carbon molecule is further desaturated and elongated until the most prominent long-chain PUFA of n-6, C20:4n-6; and n-3, eicosapentanoic acid (C20:5n-3) and C22:6n-3 are formed (reviewed by Gurr & Harwood, 1991 and Sprecher, 2000).

The final steps in the pathway of C22:5n-6 and C22:6n-3 formation independent of delta-4 desaturase were recently elucidated with *in vitro* studies using animal cells (Voss et al., 1991; reviewed by Sprecher, 2000; Figure 1.15). The synthesis of C22:5n-6 from C20:4n-6 proceeds via elongation to C22:4n-6 and then to C24:4n-6 with a delta-6 desaturase to form C24:5n-6 and partial beta-oxidation in peroxisomes to C22:5n-6 (Voss et al., 1991; and reviewed by Sprecher, 2000). The synthesis of C22:6n-3 from C20:5n-3 seems to be similar to that of the n-6 fatty acids: C20:5n-3 to C22:5n-3 to C24:5n-3 to C24:6n-3, with beta-oxidation to C22:6n-3 (Voss et al., 1991; and reviewed by Sprecher, 2000). Recently, Li et al. (2000) unequivocally demonstrated in developing piglets that microsomes and peroxisomes are both required for biosynthesis of C22:6n-3 from C18:3n-3. It is still uncertain as to the type of delta-6 desaturase and elongase enzymes required by this pathway but further research should provide an understanding of this point (Sprecher, 2000).

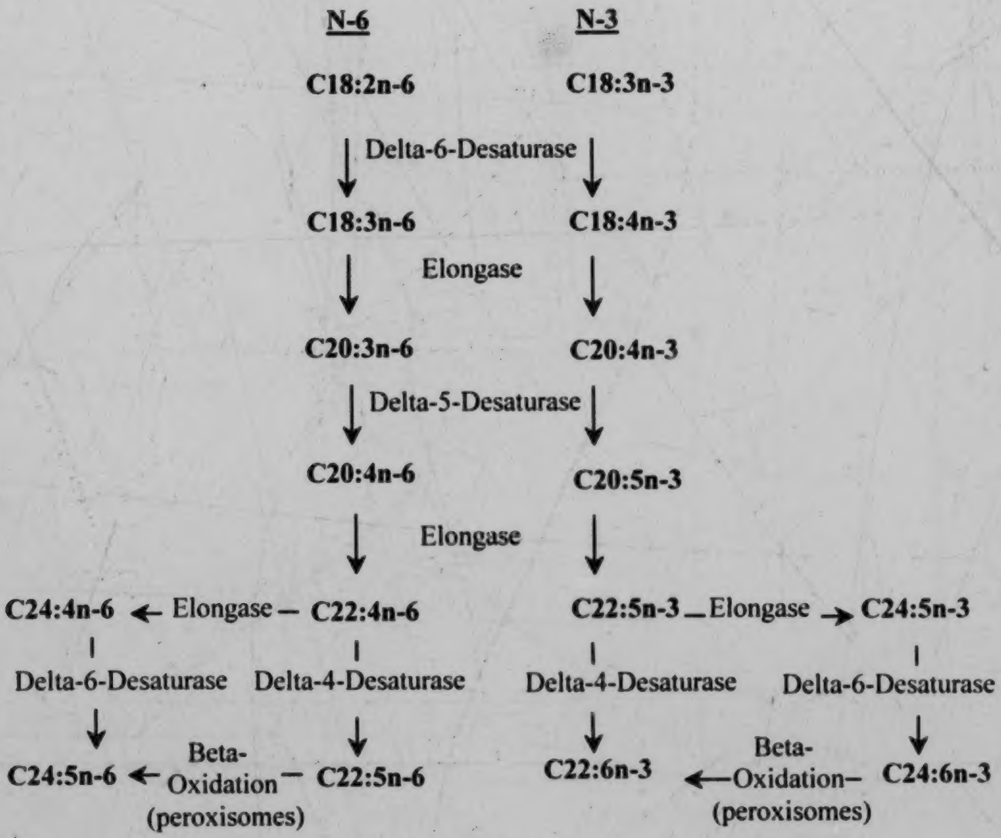


Figure 1.15 Polyunsaturated Fatty Acid Biosynthesis

a) Delta-6-Desaturase

The delta-6 desaturase has been isolated and characterized from different species, including plants (Sayonara et al., 1997), moss (Grime et al., 1998), nematodes (Napa et al., 1998), and recently human (Cho et al., 1999). Tissue distribution analysis of human delta-6 desaturase revealed that there are high mRNA levels in brain, liver, heart, and lung (Cho et al., 1999). The human delta-6 desaturase gene encodes a 444 amino acid protein which contains a membrane spanning domain, a cytoplasmic b_5-like domain and three conserved histidine-rich domains (Cho et al., 1999).

b) Delta-5-Desaturase

The delta-5 desaturase has also been isolated and characterized from several sources, including bacteria (Aguilar et al., 1998), fungus (Knutzon et al., 1998), and nematode (Watts & Browse, 1999). Recently, Leonard et al (2000) cloned and characterized delta-5 desaturase in human liver. It was found that the human delta-5 desaturase gene also encodes a 444 amino acid protein which contains an N-terminal cytochrome b_5-like domain, as well as three histidine rich domains (Leonard et al., 2000). The delta-5 and delta-6 desaturase had approximately 62% homology based on the predicted amino acid sequence encoded by the cDNA and both enzymes were localized on chromosome 11q12 (Leonard et al., 2000). The tissue expression profile of delta-5 desaturase revealed that this gene was highly expressed in fetal liver and brain, adult brain, and adrenal gland (Leonard et al., 2000). Interestingly, the level of delta-5 desaturase mRNA was six-fold greater in fetal liver than adult liver (Leonard et al., 2000).

c) Elongase

In plants and mammals, the microsomal fatty acid elongation system depends on four distinct enzymatic activities: a β-ketoacyl-CoA synthase, a β-ketoacyl-CoA reductase, a β-hydroxylacyl-CoA dehydratase and an enoyl-CoA reductase (Fehling & Mukherjee, 1991; reviewed by Cinti et al., 1992). It is assumed that the first enzyme, β-ketoacyl-CoA synthase, in this sequence determines the substrate specificity of elongase system as well as the extent of fatty acid elongation (Millar & Kunst, 1997). Several genes for the elongation enzymes have been cloned from plant (Lassner et al., 1996; Todd et al., 1999) and yeast (Toke & Martin, 1996; Oh et al., 1997). However, these elongase enzymes have been responsible for the synthesis of saturated and/or monounsaturated very long-chain fatty acids not PUFAs. Zank et al (2000) cloned and characterized a fatty acid elongase enzyme specific for n-6 PUFAs in moss, *P. patens*. The complete nucleotide sequence for the *P. patens* cDNA consisted of 1200 base pairs which contained an open reading frame of 873 base pairs encoding a protein of 290 amino acids with a calculated molecular mass of 33.4 kDa. Recently, the elongase enzyme has been isolated and characterized from yeast (Lassner et al., 1996; Millar & Kunst, 1997) and fungus (Parker-Barnes et al., 2000). Recently, Zhang et al. (2001) found a gene in humans with Stargardt-like macular dystrophy that has DNA sequence homology to yeast elongase. This gene (EL0VL4) found on chromosome 6q14 (Stone et al., 1994; Edwards et al., 1999) encodes a putative protein of 314 amino acids with approximately 35% identity to the yeast elongase protein (Zhang et al., 2001). Tissue distribution analysis of elongase mRNA showed that this gene was only expressed in retina and brain (Zhang et al., 2001). Future work on the elongase gene expression will be needed to fully elucidate their role in PUFA biosynthesis.

3. Very-Long Chain N-6 and N-3 Fatty Acids

a) Structure and Tissue Distribution

Very long chain fatty acids with carbon chain lengths >22 occur in human and rat brain (Poulos et al., 1988; Robinson et al., 1990). High levels of these fatty acids are also found in human brain with inherited peroxisomal disease, such as, Zellweger's syndrome and adrenoleukodystrophy (Poulos et al., 1986a; Poulos et al., 1988). In brain, very long chain fatty acid are polyenoic with 4, 5, or 6 double bonds and belong to n-6 and n-3 families with carbon chain lengths up to forty in brain (Poulos et al., 1986b; Sharp et al., 1991). These fatty acids occur exclusively in PC and are esterified mainly in the sn-1 position of the glycerol backbone, whereas saturated, monounsaturated, and other PUFAs are part of the sn-2 position (Aveldano, 1988; Poulos et al., 1988; Robinson et al., 1990). This positioning contrasts to that typical of most membrane phospholipid species in other tissues (Poulos et al., 1988). In normal brain, the polyenoic very long chain fatty acids represent a minor component of the total fatty acid fraction (<1%) but is increased in brain with Zellweger's syndrome, and inherited metabolic disease characterized by peroxisomal deficiency (Poulos et al., 1986a and 1986b and 1988). The fatty acid composition of the very long chain polyenoic fatty acids is also different in normal and diseased brain. Tetra- and pentaenoic n-6 derivatives are the major 32-38 carbon fatty acids in normal brain, whereas Zellweger patients contain predominately pentaenoic and hexaenoic acids (Poulos et al., 1988; Sharp et al., 1991). In normal rat brain, these fatty acids belong to both n-6 and n-3 series up to 38 carbons (Robinson et al., 1990). Johnson et al. (1992) reported the existence of very long chain monoenoic fatty acids in human brain up to 28 carbons in chain length. These fatty acids contain predominately two positional isomer series, the n-7 and n-9 cis homologues (Johnson et al., 1992). Robinson et al (1990) speculated that this unique brain lipid may be required for the correct orientation of integral membrane proteins.

b) Biosynthesis

Synthesis of polyenoic very long chain fatty acid has been shown to occur in a number of different mammalian tissues using either radiolabeled polyenoic fatty acids or acetate as precursors. Labeled polyenoic very long chain fatty acids are formed from [^{14}C] acetate and [^{14}C] C20:4n-6, respectively, by isolated rat spermatocytes and spermatids (Grogan & Lam, 1982; Grogan & Heath, 1983). [^{14}C] C20:4n-6 injected into rat testis was also elongated in vivo to form n-6 tetra- and pentaenoic very long chain fatty acids with up to thirty carbon atoms (Grogan, 1994). Very long chain polyenoic fatty acids have been shown to be synthesized by elongation from shorter chain polyenoic fatty acids in brain (Figure 1.16; Robinson et al., 1990). Also, the presence of n-6 hexaenoic very long chain fatty acids in brain suggests some desaturation of PUFAs with >24 carbon atoms may also occur (Poulos et al., 1986a; Sharp et al., 1991).

Intracerebral injection of [1-^{14}C] C26:4n-6 into rats was elongated to form polyenoic very long chain fatty acids with up to 36 carbons (Robinson et al., 1990). Also, significant amounts of label were detected in C26:5n-6 and C28:5n-6, indicating that desaturation of the 26 carbon fatty acids can take place in brain (Robinson et al., 1990). Recently, Suh et al. (2000) has shown that long chain PUFAs are involved in the synthesis of very long-chain polyenoic fatty acids in retina. It was found that C20:5n-3 but not C22:6n-3 injected into the vitreal fluid of the eye of rats was a substrate for formation of very long chain polyenoic fatty acids. The C22:5n-3 fatty acid derived from C20:5n-3 was highly labeled and was detected in the pentaenoic and hexaenoic very long chain fatty acids while greater than 90% of the injected ^{3}H-C22:6n-3 into the eye remained as C22:6n-3. Therefore, this study suggests that long chain PUFAs particularly, C20:5n-3 and C22:5n-3 can synthesize very long chain polyenoic fatty acids in the retina. However, it is still not known whether there are separate elongases or individual elongases specific for the very long chain n-6 and n-3 fatty acids (Poulos et al., 1986a).

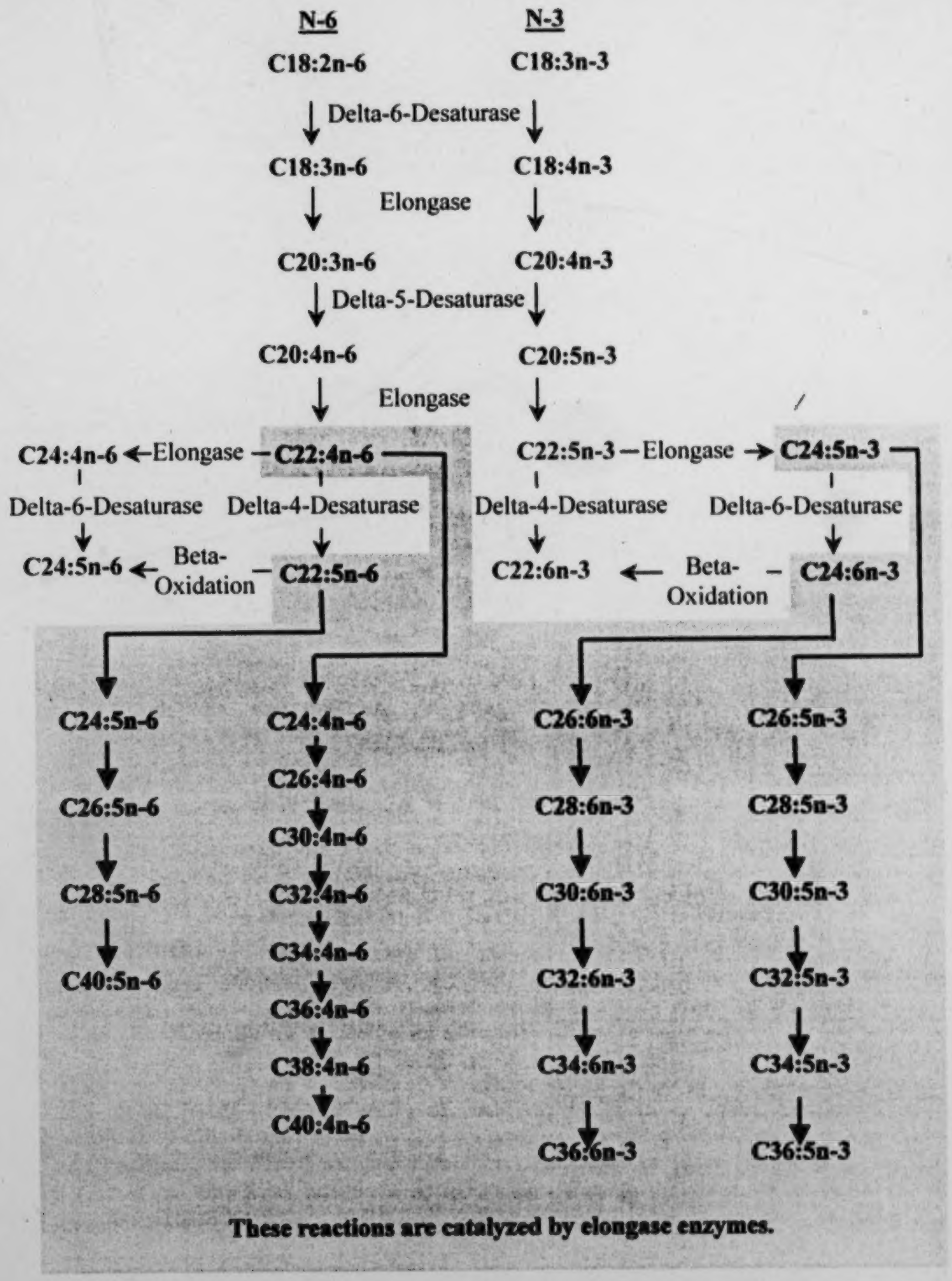


Figure 1.16 Very Long Chain N-6 and N-3 Fatty Acid Biosynthesis

c) Biological Function

The physiological role of the polyenoic very long chain fatty acids is not known. Recently, the possible involvement of very long chain fatty acids in membrane signal transduction has been explored in rat brain (Hardy et al., 1994). C32:4n-6 and C34:6n-3 activates purified rat brain protein kinase C in vitro with maximal activity being between 25 and 50 uM (Hardy et al., 1994). The same effect was also observed by the same authors when long-chain fatty acid (C20:4n-6, C20:5n-3, or C22:6n-3) were used (Hardy et al., 1994). Therefore, it is hard to distinguish the unique role of very long chain fatty acids in brain.

4. Composition and Developmental Changes in Brain

Similar to brain phospholipids profiles during development, the fatty acid composition of these phospholipids are also distinct and exhibit changes with age. Some studies examining changes in fatty acyl constituents of phospholipids during development have analyzed total phospholipids, while others analyzed individual phospholipids which is a weakness in brain lipid research because of the selectivity of individual phospholipids for specific fatty acids.

a) Total Phospholipids

Crawford et al. (1977) examined human fetuses and infants up to two years of age. The study found that 60-70% of adult long-chain polyene levels were accumulated at birth. After this period, the rate of accumulation of these fatty acids declines. Changes between the types of long-chain polyenoic families in human brain were different (Martinez et al., 1974).

Biran & Bartley (1964) and Kishimoto et al. (1965a) studied the fatty acid composition of total phospholipids in developing rodent brain. They suggested that some fatty acid ratios could be correlated to brain maturity. For example, the C16:0/C18:0 ratio drops with increasing age while C18:1/C18:0 increases. Sinclair and Crawford (1972a) correlated myelination in brain to the accumulation of C20:4n-6 and C22:6n-3 in gray matter and C24:1 and C20:1 in myelin.

b) Individual Phospholipids

In human brain, Svennerholm et al. (1964, 1965, 1968, 1973, 1978a, & 1978b) reported the fatty acid composition of individual phospholipids with varying ages.

i) Phosphatidylcholine

Differences in the acyl group profiles of PC are found in cerebral cortex during development (Svennerholm, 1968). Fatty acids of the n-6 series, mainly C20:4n-6 are low during the fetal period but increase shortly after birth (Svennerholm & Vanier, 1973)

ii) Phosphatidylethanolamine

In the cerebral cortex of human brain, the PE C20:4n-6 and C22:4n-6 decline with age, whereas the n-3 series C22:5n-3 and C22:6n-3 increase with age (Svennerholm, 1968; Svennerholm & Vanier, 1973).

iii) Phosphatidylserine

In human brain cortex, the PS C20:4n-6 and C22:4n-6 content increase during fetal development to term and then decrease with age (Svennerholm, 1968; Svennerholm & Vanier, 1973). On the other hand, C22:6n-3 increases with age (Svennerholm, 1968; Svennerholm & Vanier, 1973).

iv) Phosphatidylinositol

The fatty acid composition of PI in human brain during fetal and postnatal maturation into aging has been reported (Svennerholm, 1968; Svennerholm & Vanier, 1973). PI is characterized by high levels of C20:4n-6 and modest level of C22:6n-3 (Svennerholm, 1968). The C20:4n-6 content of PI increases up to four years of age in cerebral cortex (Svennerholm, 1968; Svennerholm & Vanier, 1973).

c) Sphingolipids

In general, brain sphingolipids contain less than twenty different fatty acids (Giusto et al., 1992). The fatty acids that are amide linked to the sphingosine moiety varies from twelve to over thirty carbons in chain length, but are mainly C16:0, C18:0, C22:0, C24:0, and C24:1 (O'Brien & Sampson, 1965; Stallberg-Stenhagen & Svennerholm, 1965; Rouser et al., 1972; Rouser & Yamamoto, 1972; White, 1974; Barenholz & Thompson, 1980). Sphingolipids species with polyenoic long-chain fatty acids, and with 2-hydroxy fatty acid have been described (Dawson & Vartanian, 1988; Robinson et al., 1992). Sphingolipid levels tend to increase with age in different organs, and may also vary with diet (Myher et al., 1981; Barenholz & Thompson, 1981). Dietary fat induced changes in brain sphingolipid n-6 and n-3 fatty acids composition with development are limited.

5. Accretion in Brain

Formation of neuronal membranes requires synthesis and assembly of membrane phospholipids containing significant amounts of essential fatty acid, primarily C20:4n-6 and C22:6n-3. Little quantitative evidence exists prior to twenty-four weeks of gestation in the human. Accretion of essential fatty acids during the last trimester of intrauterine development has been estimated (Clandinin et al., 1981a).

Analyses of whole-body fat content (Widdowson, 1968; Widdowson et al., 1979) indicates that preterm infants, with appropriate weight for gestational age of 1300 g at birth, have a total body fat content of about 30 g compared with the term infant of 3500 g with a total body fat content of 340 g. Clandinin et al. (1981b) estimated that approximately 2783 mg of n-6 fatty acids and 387 mg n-3 fatty acids accrue in adipose tissue each week *in utero*. For premature infants, birth after only a few more weeks of intrauterine development would dramatically increase the potential reserve of fatty acids in adipose tissue both for total fatty acids used for energy production and for essential fatty acids used for synthesis of structural tissues (Clandinin et al., 1981b). These estimates are also supported by the body of research by Van Houwelingen et al. (1992), suggesting that the growing fetus represents a large draw upon maternal essential fatty acid stores and perhaps that a limitation in the size of the maternal essential fatty acid stores may impact on fetal growth and development, particularly brain growth and development.

During the third trimester of human development, n-6 and n-3 fatty acids accrue in fetal tissues as an essential component of structural lipids and rapid synthesis of brain tissues occurs (Clandinin et al., 1980a). This rapid synthesis causes increases in cell size, cell type and cell number (Clandinin et al., 1980a). Brain lipid levels increase rapidly during this period (Clandinin et al., 1980a). Levels of C18:2n-6 and C18:3n-3 are consistently low in the brain during the last trimester of pregnancy (Clandinin et al., 1980a). However, accretion of long-chain essential fatty acid desaturation products C20:4n-6 and C22:6n-3 occur and the absolute accretion rates of the n-3 fatty acids, specifically, C22:6n-3, are greater in the prenatal period compared with the postnatal period (Clandinin et al., 1980a and 1980b). It is apparently critical that the developing fetus obtains the correct types and amounts of fatty acids to ensure complete and proper development of brain membranes (Clandinin et al., 1980a and 1980b). Timing of the availability of these fatty acids is also a factor (Clandinin et al., 1980a and 1980b). Collectively, this quantitative information indicates that large amounts of C22:6n-3 and C20:4n-6 are required during development of neural tissue when cellular differentiation and active synaptogenesis are taking place (Clandinin et al., 1980a and 1980b; reviewed by Cunnane et al., 2000).

6. Endogenous Sources for Fetal Brain

a) Placenta

The placenta is a specialized organ that provides nutrients to the fetus and exchanges metabolic waste products from the fetus (Crawford et al., 1976; reviewed by Crawford, 2000). Research has shown that all of the n-6 and n-3 fatty acids acquired by the fetus come from maternal circulation via the placenta (reviewed by Innis, 1991, Dutta-Roy, 2000, Crawford, 2000). This transfer of fatty acids from the mother to the fetus influences the composition of fatty acid in the developing tissue of the fetus especially the central nervous system (reviewed by Innis, 1991, Dutta-Roy, 2000, and reviewed by Crawford, 2000). Since glycerolipids and triglycerides can not enter the fetal circulation, hydrolysis of these lipids to free fatty acids by lipoprotein lipase on the maternal side of the placenta are required (Hummel et al., 1976; Elphick & Hull, 1977).

The long-chain products from C18:2n-6 and C18:3n-3 metabolism, specifically, C20:4n-6 and C22:6n-3 are higher in fetal plasma compared to maternal plasma with even a greater accumulation in the fetal tissue (Crawford et al., 1976; reviewed by

Crawford, 2000). This process of increased long-chain PUFAs in fetal versus maternal blood is known as biomagnification (Crawford et al., 1976; reviewed by Crawford, 2000). It has been hypothesized that this process is the result of a specific mechanism within the placenta that can sequester and release C20:4n-6 and C22:6n-3 into the fetal circulation (Crawford et al., 1976; reviewed by Crawford, 2000). This unidirectional transport of long-chain PUFAs, particularly, C22:6n-3, from maternal to fetal circulation have been shown to be driven by high-affinity C22:6n-3-specific transport proteins in the placenta (Campbell et al., 1998; reviewed by Crawford, 2000). Thus, providing the fetus with long-chain PUFAs needed for growth and development.

b) Intestine

After dietary lipids have been digested by a series of lipases, long-chain fatty acids in bile salt micelles are protonated by the acidified unstirred water layer adjacent to the brush border membrane (BBM; Isola et al., 1995). This protanation of micelles in the unstirred water layer releases the long-chain fatty acids and allows for their uptake by either fatty acid binding or fatty acid transporter proteins in the BBM (Zhou et al., 1995; Poirier et al., 1996; Abumrad et al., 1999; Stahl et al., 1999). The long-chain fatty acids in the intestinal cells will be reassembled into triacylglycerols and packaged into chylomicrons (reviewed by Thomson et al., 1997). Moreover, these chylomicrons will be transported into the lymphatics and enter the blood via the portal vein (Linscheer & Vergroesen, 1988). Once in the blood, the long-chain fatty acids can cross the placenta and go into the fetal circulation where it can be taken up by the fetal brain (Sinclair, 1975; Green & Yavin, 1993). Interestingly, a major serum protein during fetal development, alpha-fetoprotein, has been suggested to be involved in the transfer of C20:4n-6 and C22:6n-3 (Lampreave et al., 1982; Calvo et al., 1988). Garg et al. (1988) found that intestinal cells have a delta-6 and delta-9 desaturase activity. These desaturases may have a role in the modification of dietary n-6 and n-3 fatty acid, C18:2n-6 and C18:3n-3, respectively, into long-chain PUFAs that may be transported out of the enterocytes and go into the fetal circulation where it can be taken up by the fetal brain via a long-chain PUFAs-specific fatty acid binding protein (Balendirian et al, 2000). Recently, delta-5 and delta-6 desaturase mRNA were found in the small intestine of the human using quantitative polymerase chain reaction methodology (Leonard et al., 2000). However, whether or not these enzymes are fully active in premature infants have yet to be examined.

c) Liver

Recent research by Bazan & Scott (1990) has established that the liver can synthesize and transfer C20:4n-6 and C22:6n-3 via lipoproteins to the brain but this phenomenon has not been demonstrated in neonates. However, later studies by Green & Yavin (1993) and Burdge & Postle (1994) indicated that the contribution of liver to C20:4n-6 and C22:6n-3 in the brain is negligible. Therefore, indicating that placental transfer may play a significant role in providing long-chain PUFAs to the fetal brain especially during the third trimester.

d) Metabolism in Brain Cells

Many questions remain about the role of brain in synthesizing C20:4n-6 and C22:6n-3 from dietary precursors. Animal studies with radio-labeled C18:2n-6 and C18:3n-3 indicate that the brain is capable of desaturating and elongating C18:2n-6 and C18:3n-3 to C20:4n-6 and C22:6n-3, respectively (Dhopeshwarkar et al., 1971a and 1971b; Sinclair & Crawford, 1972b; Dhopeshwarkar & Subramanian, 1976; Cohen & Bernsohn, 1978; Cook, 1978; Purvis et al. 1983; Clandinin et al., 1985; Anderson & Connor, 1988). These studies of brain fail to clarify which cell types within this tissue can provide C20:4n-6 and C22:6n-3. Studies with isolated brain cells provide evidence that both neuronal and glial cells may desaturate and elongate C18:2n-6 and C18:3n-3 to C20:4n-6 and C22:6n-3, respectively (Yavin & Menkes, 1974; Dhopeshwarkar & Subramanian, 1976; Cohen & Bernsohn, 1978; Clandinin et al., 1985a; Anderson & Connor, 1988).

Microvessels and plasma contain amounts of C18:3n-3 and C20:5n-3, thus it is possible that these fatty acids could supply C22:6n-3 to brain (Edelstein, 1986; Clandinin et al., 1997). Moore et al. (1990) investigated whether cerebroendothelial cells could desaturate and elongate C18:2n-6 and C18:3n-3 to C20:4n-6 and C22:6n-3, respectively. Isolated cerebroendothelial cells were incubated with radiolabeled C18:2n-6 or C18:3n-3 and were found to take up C18:2n-6 and C18:3n-3 equally and desaturate and elongate C18:2n-6 to C22:4n-6 and C18:3n-3 to C22:5n-3 (Moore et al., 1990). The major metabolite of C18:2n-6 was C20:4n-6 and of C18:3n-3 was C20:5n-3 (Moore et al., 1990). Desaturation and elongation of C18:3n-3 by cerebroendothelial cells far exceeded that of C18:2n-6, suggesting some specificity for n-3 fatty acids (Moore et al., 1990). Delton-Vandenbroucke et al. (1997) demonstrated that cerebroendothelial cells can metabolize C22:5n-3 to C20:5n-3, C22:6n-3, C24:5n-3, and C24:6n-3. The presence of twenty-four carbon n-3 metabolites in cerebroendothelial cells lipids and culture media suggests that metabolism of n-3 fatty acids in cerebroendothelial cells were using a delta-4 desaturase independent pathway similar to that shown in rat liver (Voss et al., 1991). Thus, cerebroendothelial cells can desaturate and elongate C18:2n-6 and C18:3n-3 to C20:4n-6 and produce a small amount of C22:6n-3, respectively.

Moore et al. (1991) determined that astrocytes from rat brain can desaturate and elongate C18:2n-6 and C18:3n-3 to C20:4n-6 and C22:6n-3, respectively. Rat type I astrocytes from either cerebrum or cerebellum cultured for twelve days were incubated with radio-labeled C18:2n-6 or C18:3n-3 formed C20:4n-6 and C22:6n-3, respectively (Moore et al., 1990). In contrast, cultures of rat cerebral or cerebellum neuronal cells did not desaturate fatty acid to produce C20:4n-6 and C22:6n-3 (Moore et al., 1990). Instead, the neuronal cells appear to only elongate C18:2n-6 and C18:3n-3 (Moore et al., 1990). Hence, astrocytes, not neuronal cells, appear to synthesize C20:4n-6 and C22:6n-3 in brain. Cerebroendothelial cells and astrocytes together supply C22:6n-3 to neuronal cells (Moore, 1993). In co-cultures, astrocytes synthesize and release large amounts of C22:6n-3 from C20:5n-3 made by the cerebroendothelial cells (Moore, 1993). Neuronal cells then take up C22:6n-3 released from astrocytes and incorporate fatty acid into neuronal cell plasma membranes (Moore, 1993). Thus, cerebroendothelial cells and astrocytes seem to be needed together to synthesize C22:6n-3 for uptake by the neuronal cell during growth. These observations on the cellular partitioning of C20:4n-6 and C22:6n-3 synthesis in brain cells are based primarily on the rat and are largely undetermined for the human brain.

7. Exogenous Sources for Neonatal Brain

a) Human Milk

The n-6 and n-3 fatty acid composition of human milk is partly dependent on the maternal diet (Chappell et al., 1985). The content of C18:2n-6, C20:5n-3, and C22:6n-3, but not C20:4n-6, in breast milk responds to the quantity of these fatty acids in the mother's diet (Chappell et al., 1985). The fat in mature human milk is contained within membranes called fat globules. The core of the fat globules has 98-99% triglyceride with the membrane mainly composed of phospholipids, cholesterol, and proteins (Hamosh et al., 1987).

Human milk contains both n-6 and n-3 fatty acids (Clandinin et al., 1981a). Ten to fifteen percent C18:2n-6 and one percent C18:3n-3 are typical amounts of essential fatty acids in the breast milk fat of North American and European women (Clandinin et al., 1981a). Based on quantitative analysis of twenty-four hour milk collections, the n-6 long-chain polyenes are at 0.5-1.5% and the n-3 long-chain polyenes at 0.3-0.6% (Clandinin et al., 1981a; Jensen, 1989; reviewed by Hamosh & Salem, 1998). The long-chain polyenes in human milk is predominately C20:4n-6 for n-6 and C22:6n-3 for n-3 fatty acids (Clandinin et al., 1981a).

The fatty acid composition in human milk is not only a result of maternal dietary fat intake, but also other factors, such as, mobilization of fat from maternal fat stores (adipose tissue) and endogenous synthesis by the mammary glands (Jensen, 1989). But, C18:2n-6 and C18:3n-3 is not synthesized by the mammary gland and must be supplied by the maternal diet (Chappell et al., 1985). The synthesis of long-chain polyenes of n-6 and n-3 fatty acids might occur in the mammary gland independently of the length of gestation (Hamosh et al., 1992). However, the proportion of long-chain PUFAs to saturated fatty acids present in human milk has been shown to correspond with the ratio of polyunsaturated to saturated fatty acids in the maternal diet (Chappell et al., 1985).

Studies have shown that there are differences in the fat content of milk from mothers of term and preterm infants. Chappell et al. (1983) found that milk from mothers who delivered preterm infants showed a higher content of C18:2n-6 and C18:3n-3 and their long-chain polyenoic homologues compared to term mothers. Furthermore, the level of their long-chain polyenoic homologues were shown to be significantly higher in colostrum and milk of mothers of premature infants than mothers delivering full term infants (Bitman et al., 1983). Luukkainen et al. (1994) studied the content of C20:4n-6 and C22:6n-3 in human milk of mothers who delivered preterm and term infants from one week to six months of lactation. The results from this study demonstrated that the concentrations of C20:4n-6 and C22:6n-3 in human milk after six months of lactation were higher in preterm than term infants. This suggests that human milk can provide and meet the C20:4n-6 and C22:6n-3 requirements in preterm infants when there is a reduced supply of these fatty acids to the fetus via placenta *in utero* (reviewed by Cunnane et al, 2000).

b) Infant Formulas

Human milk provides the optimal source of nutrition for infants to at least three months of age (FAO Expert Committee, 1977). Hence, it is conceivable that formulas intended for feeding infants for whom mother's milk is not available should mimic the composition of human milk as closely as possible. Special formulas have been designed to provide adequate intake of calories, proteins, carbohydrates, fluids, vitamins, and minerals, necessary to support the rapid growth and development of the infant (Reichman et al., 1981; Garza et al., 1987). The fat content of infant formulas whether cow's milk or soy protein based is provided by one or more vegetable oils (Jensen et al., 1978; Jensen, 1989). However, the vegetable oils used in these infant formulas do not contain n-6 and n-3 long-chain PUFAs (Clandinin et al., 1980a and 1980b). Research over the past three decades has focussed on the essential fatty acid requirements of preterm infants and the quantities of C18:2n-6, C18:3n-3, C20:4n-6, C20:5n-3, and C22:6n-3 that should be added to infant formulas. Although dietary intake of 1-2 % kilocalories for C18:2n-6 prevents and avoids signs of essential fatty acid deficiency in infants, such low intake of C18:3n-3 can not support adequate accretion of C22:6n-3 in the neonate (Clandinin et al., 1980a and 1980b). Infant formulas supplemented with C20:4n-6 and C22:6n-3 produce a clear dose response in the content of C20:4n-6 and C22:6n-3 in erythrocyte total plasma membrane phospholipids with 0.6% C20:4n-6 and 0.4% C22:6n-3 in the formula fat providing sufficient amounts of these fatty acids to achieve a fatty acid composition of C20:4n-6 and C22:6n-3 similar to that of infants fed human milk (Clandinin et al., 1992;

Clandinin et al., 1997). Recently, Clandinin et al. (1999) determined the magnitude of the plasma essential fatty acid pools in individual lipoprotein fractions of preterm infants fed commercial formula devoid of C20:4n-6 and C22:6n-3 and compared this with similar infants fed formulas containing a range of 0.32-1.1% C20:4n-6 and 0.24-0.75% C22:6n-3 of the total fatty acids in the formula. The results of the study revealed that on a quantitative basis the phospholipid fraction of lipoproteins contain >75% of the C20:4n-6 and C22:6n-3 in plasma (Clandinin et al., 1999). Moreover, high density (HDL) and low density (LDL) lipoprotein phospholipids and cholesterol esters contained the largest pool of C20:4n-6 and C22:6n-3 in plasma lipoproteins (Clandinin et al., 1999). Infants fed a formula without C20:4n-6 and C22:6n-3 showed a reduction in C20:4n-6 level in the phospholipid fraction of all lipoprotein and HDL and LDL cholesterol ester fraction (Clandinin et al., 1999). Supplementing infant formulas with increasing levels of C20:4n-6 and C22:6n-3 produced a clear dose response in the level of C20:4n-6 found in the HDL and LDL phospholipid fraction (Clandinin et al., 1999). From comparison of the fatty acid levels present in the lipoproteins it appears that a formula level of 0.49% C20:4n-6 and 0.35% C22:6n-3 provides sufficient levels of these fatty acids to achieve a similar fatty acid content to that of infants fed breast milk for the major lipoprotein fraction (Clandinin et al., 1999).

Many international advisory committees, such as, European Society of Pediatric Gastroenterology and Nutrition (1991) (ESPGAN), British Nutrition Foundation (1992) (BNF), International Society for the Study of Fatty Acids and Lipids (1994) (ISSFAL), and Food and Agriculture Organization (FAO)/ World Health Organization (1994) (WHO) have recommended that infant formulas be supplemented with n-6 and n-3 long-chain PUFAs. However, despite these recommendations, a few countries continue to use formulas based on vegetable oils. In North America, there are no commercial formulas that contain polyunsaturated fatty acids greater than eighteen carbons in chain length (Raiten et al., 1998; Clandinin, 1999). Therefore, it is important to establish if infants possess the enzymes and their level of activities for elongation and desaturation of long-chain PUFAs that are required for growth and development of the infant.

H. FACTORS AFFECTING NEURAL TISSUE N-6 AND N-3 FATTY ACID COMPOSITION AND FUNCTION

1. Diet

Brain membranes were generally viewed as resistant to structural change by both endogenous and exogenous factors. Data has shown that brain membranes are much more sensitive to changes in composition induced by dietary fat than previously thought (Jope & Jenden, 1979; Wurtman et al., 1981; Foot et al., 1982; Lee, 1985; Bourre et al., 1989a; reviewed by Clandinin, 1999). Moreover, the extent of the changes in brain membrane composition by dietary fat varies between brain regions, cell types, and organelles (reviewed by Hargreaves & Clandinin, 1990, Clandinin et al., 1991 and Clandinin et al., 1997).

Earlier studies examining the role of dietary fat on brain membrane composition have used rodents that were fed C18:2n-6 deficient diets for several weeks to a few generations. Results from these studies demonstrate qualitative changes in brain membrane fatty acid composition associated with essential fatty acid deficiency (i.e. increase in C20:3n-9 and decrease in C20:4n-6; Paoletti & Galli, 1972; Sun & Sun, 1974).

By feeding nutritionally adequate diets, dietary intake of C18:2n-6, C18:3n-3, or the proportion of C18:2n-6 to C18:3n-3, particularly during development, has been shown to influence the content of PUFAs in membrane lipids by changing the composition of whole brain, oligodendrocytes, myelin, astrocytes; mitochondrial, microsomal, synaptosomal membrane, and recently neurites (axons and dendrites) (Lamptey & Walker, 1976; Tahin et al., 1981; Foot et al., 1982; Bourre et al., 1984; Hargreaves & Clandinin, 1989; Innis & de la Presa Owens, 2001). Feeding diets with a C18:2n-6 to C18:3n-3 fatty acid ratio between four to one to seven to one to rats from birth to one, two, three, and six weeks of age indicates that diet alters neuronal and glial cell membrane fatty acid composition differently, and in a region and time specific manner (Jumpsen et al., 1997a and 1997b).

The functional effect of diet-induced changes in the fatty acid composition of membrane phospholipids in brain has been the subject of some research (reviewed by

Stubbs & Smith, 1984; Lee et al., 1986; Salem et al., 1988). Dietary fat induced change in membrane fatty acid composition can affect membrane function by modifying membrane fluidity and thickness, lipid phase properties, polar lipid composition or specific interactions with membrane proteins (Clandinin et al., 1985b; Litman & Mitchell, 1996; reviewed by Clandinin et al., 1997 and Fernstrom, 1999). Previous studies showed that deficiency of n-3 fatty acid in the diet changed membrane physical properties (Park & Ahmed, 1992: Yoshida et al., 1997; Huster et al., 1998), membrane-bound enzymes (Bourre et al., 1989; Vaidyanathan et al., 1994; Tsutsumi et al., 1995; Martin, 1998), membrane channels (Poling et al., 1995; Hamano et al, 1996), receptor activity (Delion et al, 1994), and neurotransmission (Chalon et al., 1998). However, these changes induced by dietary n-3 deficiency do not reflect an overall change in membrane fluidity but rather indicate selective changes in the microenvironment of the membrane-bound proteins (Dratz & Deese, 1986; Salem et al., 1988). Many membrane-bound proteins have a specific requirement for the annular lipids surrounding them (reviewed by Yeagle, 1989). The annular lipids allow the membrane protein to achieve an active conformation for function in brain (Capaldi, 1977; Tanford, 1978; Marinetti & Cattieu, 1982; reviewed by Yeagle, 1989). Hence, any changes in these annular lipids by dietary fat may influence the function of proteins in the membrane. Several studies have shown that diets, in the absence of essential fatty acid deficiency, containing various dietary fats change the activities of membrane-bound enzymes, receptors, and carrier-mediated transport (reviewed by Clandinin et al., 1997). It is conceivable that these changes in membrane function by dietary fat can have an impact on brain function.

Animal studies have shown that when rodents or monkeys were maintained on a n-3 deficient diet, electroretinogram abnormalities (Benolken et al., 1973; Bourre et al., 1989b; Weisinger et al., 1996a), reduced visual acuity (Neuringer et al., 1984, 1986), altered stereotyped behavior (Reisbick et al., 1994), and decreased level of learning and memory occur (Lamptey & Walker, 1976; Yamamoto et al., 1987, 1988; Mills et al., 1988; Bourre et al., 198[illegible]). Dietary n-3 fatty acid deficiency affects brain functions of preterm infants as measured by cortical visual evoked potential, electroretinograms and behavioral testing of visual acuity (Uauy et al., 1990; Birch et al., 1992; Carlson et al., 1993a). Human term and preterm infants fed infant formulas without C22:6n-3 were also shown to have abnormal electroretinograms, as well as, decreased visual acuity compared to infants fed formulas containing C22:6n-3 (Uauy et al., 1990; Carlson et al., 1993a; Birch et al., 1998; Hoffman et al., 2000). Furthermore, infants fed formulas with no C22:6n-3 had lower cognitive scores compared to infants fed formulas with C22:6n-3 (Lucas et al., 1992; Carlson et al., 1994; Makrides et al., 1995; Agostoni et al., 1995; Werkman & Carlson, 1996; Willatts et al., 1998; Birch et al., 2000). The mechanism for these changes in membrane proteins and brain function induced by diet fat is not known.

2. Gene Expression of Desaturases and Elongases

The content of n-6 and n-3 fatty acids in neural tissues may be influenced by the expression of delta-6 and delta-5 desaturase and elongase enzymes. Recent cloning and characterization of delta-6 and delta-5 desaturase and elongase enzymes have allowed for analysis of their content and distribution in different tissues. Using the mouse cDNA for delta-6 desaturase, it was found that the level of delta-6 desaturase mRNA in human brain was several-fold higher than other tissues, such as, liver and lung (Cho et al., 1999). Similarly, delta-5 desaturase was expressed in human brain at comparable levels to delta-6 desaturase (Leonard et al., 2000). Recently, Northern blot analysis with a probe for elongase revealed the presence of EL0VL4 mRNA in only human retina and brain. The level of elongase mRNA was higher in retina than brain (Zhang et al., 2001). The expression of mRNA for delta-6 and delta-5 desaturase and elongase enzymes in human brain is consistent with >30% of the total fatty acids in brain phospholipids being PUFAs (Martinez, 1992). However, future work will be needed to determine whether gene expression of delta-6 and delta-5 desaturase and elongase enzymes in neural tissue is correlated and regulated by n-6 and n-3 PUFA content.

3. Phenylketonuria

Phenylketonuria (PKU) is an inborn error of phenylalanine (PHE) metabolism that occurs once in approximately seven thousand to one in fifteen thousand live births (Bickel et al., 1981; Elsas & Acosta, 1994). PKU is inherited as an autosomal recessive disorder and occurs in all ethnic groups (reviewed by Eisensmith & Woo, 1992 and Eisensmith et al, 1992). One in fifty people is a heterozygous carrier of this disease

(Pietz, 1998). PKU is caused by a deficiency or absence of phenylalanine hydroxylase (PAH) (Enzyme commission 1.14.16.1), which converts PHE, an essential amino acid in humans, to tyrosine (Jervis, 1953; Hsia, 1966; Guttler et al., 1987). The deficiency or absence of PAH causes a metabolic block in PHE catabolism which results in accumulation of PHE and reduced levels of tyrosine plasma and urine (Jervis, 1940). In untreated PKU, plasma concentrations of PHE are sufficiently high such that alternate pathways of PHE metabolism are activated leading to formation of PHE metabolites, such as, phenylpyruvate, phenylacetate, phenylacetylglutamine, and phenyllactate (Bowden & McArthur, 1972). High levels of these metabolites if not immediately diagnosed and treated in infancy can cause severe central nervous system abnormalities (Okano et al., 1991; Burgard et al., 1996).

a) Phenylketonuria causes loss of brain myelin

In both animal models and human PKU, there is defective myelination (Alvord et al., 1950; Prensky et al., 1971; Shah et al., 1972; Loo et al., 1978; Lane et al., 1980). The reduced amount of myelin in hyperphenylalanemic (HPH) rats and brain of PKU individuals was either caused by inhibition of myelin synthesis or increased myelin degradation. It was demonstrated in HPH rats using radio-labeled lysine that myelin turnover was increased compared to healthy control rats (7 days for HPH rats versus 17 days for control rats) (Hommes, 1982). Hommes et al. (1982) showed that the increased turnover of myelin in HPH rats was due to the fast component of myelin (the myelin fraction that has the shortest half-life) and was not due to inhibition of myelin synthesis. Taylor & Hommes (1983) confirmed increased myelin turnover in HPH rats but also showed that this increase in myelin turnover in HPH rats continued at later stages of brain development. Hence, the increase turnover of myelin in HPH rats is not compensated by an increased rate of myelin synthesis, leading to a net loss of myelin (Hommes, 1985). Thus, HPH in rats causes reduced amounts of brain myelin that is a result of increased myelin degradation.

b) Phenylketonuria causes a loss of brain fatty acids

i) Monounsaturated Fatty Acids

In human PKU, the content of monounsaturated fatty acids (MUFAs) is decreased in brain phospholipids, although the extent of the reduction varies for each type of phospholipids. In brain from autopsied PKU individuals, the amount of MUFAs in myelin PE and PC was decreased by 7% and 15%, respectively, compared to non-PKU individuals (Shah, 1979). PS and PI MUFA content from myelin of PKU individuals was reduced by 28% (Shah, 1979). The MUFA content of sphingolipids in myelin, SM and cerebrosides, was reduced by 31% and 11% compared to non-PKU individuals (Shah, 1979). In synaptosomes, oleic acid is the major MUFA present in phospholipids (Sun, 1973). The amount of oleic acid in PC of synaptosomes in PKU subjects was reduced by 12% compared to non-PKU subjects (Shah, 1979). The MUFA content in synaptosomes PE, PS and PI, and SM were also decreased by PKU (Shah, 1979). Therefore, the content of MUFAs in myelin and synaptosome phospholipids and sphingolipids are reduced in PKU.

ii) Polyunsaturated Fatty Acids

The PUFA content of each phospholipid in myelin is different and the values for the proportion of PUFA in the same phospholipid fatty acid component varies widely (O'Brien & Sampson, 1965). PUFA, particularly, C20:4n-6 and C22:6n-3 are predominant in myelin phospholipids, especially PE and PS (O'Brien & Sampson, 1965). The content of PUFA in brain of autopsied PKU individuals are significantly reduced compared to non-PKU individuals (Shah, 1979). In myelin phospholipids, the content of PUFA in PE, and PS and PI is reduced by 15% and 9%, respectively, compared to non-PKU individuals (Shah, 1979). The synaptosome PUFA content is also significantly reduced in PKU. In PC of synaptosome, the PUFA content was decreased by 4% and in PE by 19% in PKU versus non-PKU individuals. The greatest decrease in PUFA content in synaptosome phospholipids of PKU was in PS and PI which was approximately 33% (Shah, 1979). However, since the content of PE in synaptosome is greater than PS and PI combined, the reduction in PUFA , mostly C20:4n-6 and C22:6n-3, is more pronounced in PE of synaptosome (Johnson et al, 1977). Thus, the finding of these studies demonstrate that the content of PUFAs is reduced in brain myelin and synaptosome of PKU.

The reason for the low content of MUFAs and PUFAs in PKU compared to normal brain could be due to the metabolites of phenylalanine, which has been shown to inhibit substrates, and enzymes that are important for MUFA and PUFA biosynthesis. The formation of phenylacetate in PKU brain has been shown to use acetyl-CoA generated in brain (Loo et al, 1976). The depletion of acetyl-CoA in brain by the formation of phenylacetate inhibits the synthesis of fatty acids in brain (Loo et al, 1985). This inhibition of fatty acids in brain by phenylacetate may contribute to the low levels of MUFAs and PUFAs observed in PKU. A study by Shah & Johnson (1975) has shown that two other metabolites of phenylalanine, phenylacetate and phenyllactate, cause in vitro inhibition of brain desaturase activity. Elevated levels of phenylalanine have also been shown to interfere with the availability of nicotinamide adenine dinucleotide, which is a cofactor for fatty acyl CoA desaturase (Shah et al, 1970; Glazer & Weber, 1971). Thus, MUFAs and PUFAs are decreased in brain of PKU individuals by inhibition of enzymes involved in their biosynthesis.

I. CHAPTER SUMMARY

The studies presented in this chapter indicate that n-6 and n-3 fatty acids are important for brain growth and development and that alterations in dietary n-6 and n-3 fatty acids can alter brain membrane composition and function. However, the question of whether high levels of C18:2n-6 and C18:3n-3 can be synthesized to C20:4n-6 and C22:6n-3, respectively, and if providing C18:2n-6 and C18:3n-3 or their long-chain homologues in the diet will have an effect on brain structure and function still persists. Therefore, the purpose of this thesis is to investigate whether dietary n-6 and n-3 fatty acids, specifically, C18:2n-6 and C20:4n-6; and C18:3n-3 and C22:6n-3 can alter brain structure and function. Sprague-Dawley rats will be used as an animal model in this thesis because of similar brain growth and development patterns to that of humans. Thus, the results from this thesis will provide further understanding as to the role of the quality of dietary fat on brain structure and function.

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CHAPTER II. RESEARCH PLAN

A. RATIONALE

Early studies on brain morphology by Dobbing and Sands (1979) demonstrated that brain growth is non-linear and occurs in "spurts" which, depending on species may be either before or after birth. Also, studies by Dobbing and Sands (1979) have led to the concept of precocial (neurologically mature at birth e.g. sheep and guinea pig) and non-precocial (neurologically immature at birth e.g. man and rat) species (Figure 2.1). In this context, the rat provides a useful animal model for human brain development providing that account is taken of different gestational time periods. Thus, the use of two and five-week old rats in this study is adequate to mimic the pattern of brain development in preterm infants which occurs postnatally (Dobbing & Sands, 1979; Figure 2.1).

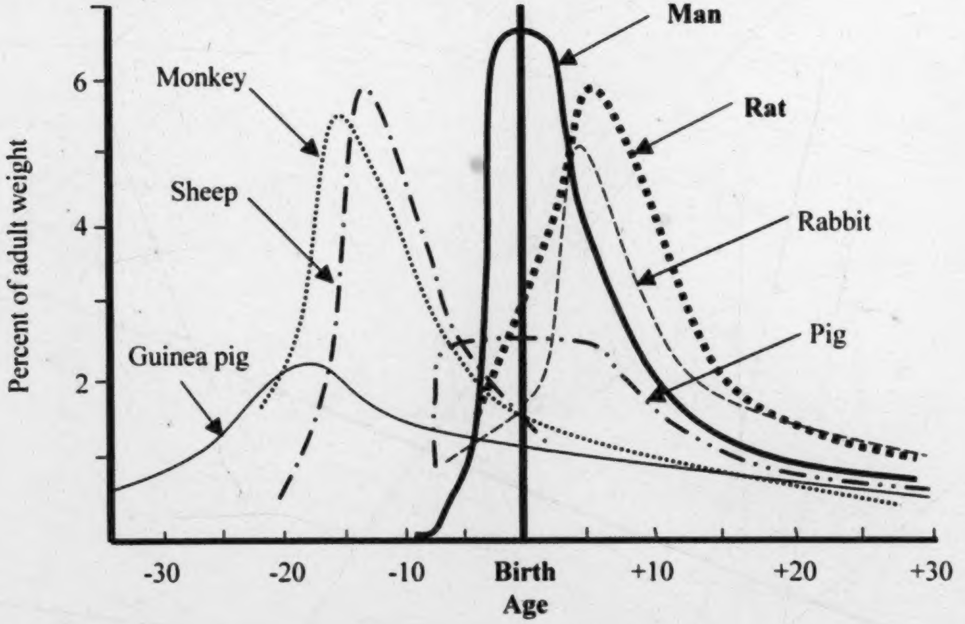

Figure 2.1 Brain Growth as a Function of Age in Different Species (expressed as increase in brain weight as percentage of the adult brain weight) as a function of age where the units of time are: rat and guinea pig, days; rabbit, 2 days; monkey, 4 days; sheep, 5 days; pig, weeks; man, months (Adapted from Dobbing & Sands, 1979)

Most research in infant nutrition investigating the effects of diets with or without C20:4n-6 and/or C22:6n-3 has shown compositional differences in the C20:4n-6 and/or C22:6n-3 content of membrane phospholipids. However, no study to date has examined whether diets with or without C22:6n-3 can increase the C22:6n-3 content of neuronal and glial cell phospholipids from whole brain of rats. It is not known whether a high intake of C18:3n-3 or a low ratio of C18:2n-6 to C18:3n-3 will result in increased C22:6n-3 accumulation in neuronal and glial cell phospholipids from rat whole brain. The metabolic fate of C18:3n-3 in the whole body of developing rats is also not known.

The SPM plays a very important role in communication between brain nerve cells, and contains high levels of phospholipids (Cotman et al., 1971; Breckenridge et al., 1973; DeGeorge et al., 1991; Jones et al., 1997). However, there is little information pertaining to the effects of dietary C20:4n-6 and C22:6n-3 on SPM phospholipid content and fatty acid composition in developing rats.

Although gangliosides are enriched in the SPM (Hakomori & Igarashi, 1993). It is not known if C20:4n-6 and C22:6n-3 are present as the fatty acyl moiety of rat SPM gangliosides. It is also not known if dietary fat can alter the C20:4n-6 and C22:6n-3 content of rat SPM gangliosides.

Phospholipids and sphingolipids are important for cell membrane structure and functions (Singer & Nicholson, 1972). Membrane-bound enzymes, such as, Na, K-ATPase which are particularly located in the SPM, play a key role in neuronal conduction in brain (Skou, 1967). Previously, Tsutsumi et al. (1995) showed that rats fed C18:3n-3 deficient diets had significantly lower brain Na, K-ATPase activity (Vmax) than rats fed diets with adequate C18:3n-3. The affinity (Km) of Na, K-ATPase for ATP was not significantly different between rats fed diets with or without C18:3n-3 (Tsutsumi et al., 1995). However, current knowledge of the effects of dietary fat with C20:4n-6 and C22:6n-3 and age on SPM Na, K-ATPase kinetics in rats is limited.

Past research has shown that sphingolipid metabolites, ceramide and sphingosine, play an important role in biological functions, such as cell differentiation, proliferation, apoptosis and signal transduction (reviewed by Merrill et al., 1997). Since sphingolipds, particularly, SM and gangliosides are concentrated in the SPM, it would be of interest to determine whether membrane-bound CDase activity is present in the SPM. Thus, studying the effects of diets with or without C18:3n-3 and/or C20:4n-6 and C22:6n-3 on brain membrane composition and function of rats may provide additional information on the role of these fatty acids in brain.

B. HYPOTHESES

Dietary fat will induce changes in phospholipid content, phospholipid and ganglioside fatty acid composition in developing brain. Specifically, dietary C20:4n-6 and C22:6n-3 will increase the C20:4n-6 and C22:6n-3 content of rat brain phospholipids with subsequent increases in SPM Na, K-ATPase activity.

It is specifically hypothesized that:

1. Feeding a high C18:3n-3 diet will not increase the C22:6n-3 content in neuronal cell phospholipids from whole brain of rats at two weeks of age.

2. Feeding a high C18:3n-3 diet will increase the C18:3n-3 content in whole body and tissue lipids of rats at two weeks of age.

3. Feeding diets with C22:6n-3 will increase the content of C22:6n-3 in neuronal and glial cell phospholipids from whole brain of rats at two weeks of age.

4. Feeding a diet with C20:4n-6 and C22:6n-3 will increase the total and individual phospholipids and decrease cholesterol content of the SPM of rats at two and five weeks of age.

5. Feeding a diet with C20:4n-6 and C22:6n-3 will increase the C20:4n-6 and C22:6n-3 content in phospholipids of the SPM of rats at two and five weeks of age.

6. The Km and Vmax of Na, K-ATPase will increase in the SPM of rats from two to five weeks of age.

7. Feeding a diet with C20:4n-6 and C22:6n-3 will not alter the Km but increase the Vmax of Na, K-ATPase in the SPM of rats at two and five weeks of age.

8. C20:4n-6 and C22:6n-3 are present as the fatty acid moiety of SPM gangliosides and feeding dietary fat will alter the C20:4n-6 and C22:6n-3 content of individual gangliosides in the SPM of two-week-old rats.

9. Alkaline CDase is present in the SPM of rat pups at two weeks of age.

C. CHAPTER FORMAT

The hypotheses posed are tested in a sequence of experiments. These experiments are organized in chapters as follows:

Chapter III test hypothesis 1.

The effect of providing a high dietary C18:3n-3 level on the C22:6n-3 content of neuronal cell phospholipids from whole brain is determined using the control diet with C18:2n-6 or C18:3n-3 added. The 20% (wt/wt) fat diets supplying C18:3n-3 from 1.6% (C18:2n-6/C18:3n-3 =21.6) to 17.5% (C18:2n-6/C18:3n-3 =1.0) of the total fatty acids are fed to the dams at parturition. The rat pups received the C18:3n-3 diets from the dam's milk from birth to two weeks of age.

Chapter IV tests hypothesis 2.

The effects of feeding a high C18:3n-3 diet on the C18:3n-3 content in whole body and tissue lipids are determined using the control diet with C18:2n-6 or C18:3n-3. The 20% (wt/wt) fat diets supplying C18:3n-3 from 1.6% (C18:2n-6/C18:3n-3 =21.6) and 17.5% (C18:2n-6/C18:3n-3=1.0) of the total fatty acids are fed to the dams at parturition. The rat pups received the same diet fat treatments from the dam's milk from birth to two weeks of age.

Chapter V tests hypothesis 3.

The effect of dietary C22:6n-3 on the neuronal and glial cell phospholipid C22:6n-3 content from rat whole brain is examined. The 20% (wt/wt) fat diets are formulated to reflect the fatty acid composition of a conventional infant formula (SMA) providing a C18:2n-6 to C18:3n-3 ratio of 7.8:1 (control diet). The C18:2n-6 to C18:3n-3 ratio of 4:1 diet has been made by adding C18:3n-3 to the control diet. Diet with 0.7% C22:6n-3 has been made by adding this fatty acid to the control diet. The rat pups received the same diet fat treatments from the dam's milk from birth to two weeks of age.

Chapter VI tests hypothesis 4, 5, 6, and 7.

The effects of feeding diets with or without C20:4n-6 and C22:6n-3 on the total and individual content of phospholipids and cholesterol (hypothesis 4); the C20:4n-6 and C22:6n-3 content of phospholipids (hypothesis 5); the kinetics of Na, K-ATPase with age (hypothesis 6); and diet treatment (hypothesis 7) are measured using control, low C18:3n-3, and C20:4n-6 and C22:6n-3 diets. The low C18:3n-3 diet has been made by adding safflower oil (C18:2n-6) as the fat blend. The C20:4n-6 and C22:6n-3 diet has been made by adding these two fatty acids to the control diet. The rat pups received the same diet fat treatments from the dam's milk from birth to two weeks of age. After weaning, the rat pups will be fed the same diets as their dams to five weeks of age.

Chapter VII tests hypothesis 8.

The presence of C20:4n-6 and C22:6n-3 in SPM gangliosides and the effects of feeding diets with or without C20:4n-6 and C22:6n-3 on SPM ganglioside fatty acid content are determined using control, low C18:3n-3, and C20:4n-6 and C22:6n-3 diets. The low C18:3n-3 diet has been made by adding safflower oil (C18:2n-6) as the fat blend. The C20:4n-6 and C22:6n-3 diet has been made by addition of these two fatty acids to the control diet. The rat pups received the same diet fat treatments from the dam's milk from birth to two weeks of age.

Chapter VIII tests hypothesis 9.

The presence of alkaline CDase in the SPM will be determined with rat pups fed a maternal diet with a C18:2n-6 to C18:3n-3 fatty acid ratio of 7.8:1 from birth to two weeks of age.

Chapter IX provides a general summary, conclusions, implications and suggestions for future research

Chapter I.

(Selected sections from this chapter have been published in)

Clandinin, M. T., Bowen, R. A. R., & Suh, M. (2001). Impact of Dietary Essential Fatty Acids on Neuronal Structure and Function. In *Neuronal Membrane to Physiological and Behavioral Function*. Humana Press (in press)

Chapter III.

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Chapter IV.

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Chapter VI.

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CHAPTER III. DOES INCREASING DIETARY C18:3N-3 ACID CONTENT INCREASE THE C22:6N-3 CONTENT OF PHOSPHOLIPIDS IN NEURONAL CELLS OF NEONATAL RATS?

A. INTRODUCTION

C20:4n-6 and C22:6n-3 are among the most abundant fatty acids in the central nervous system phospholipids. These fatty acids are found in high concentrations in brain SPM and in photoreceptor cells (Sinclair et al., 1972; Anderson et al., 1974; Fliesler & Anderson, 1983). C20:4n-6 plays an important role as a precursor of biologically active molecules like prostanoids, leukotrienes, and other lipoxygenase products (Kinsella et al., 1990). C22:6n-3 is involved in providing a specific structural environment within the phospholipid bilayer that influences important membrane functions, such as, ion or solute transport, receptor activity, and adenylate cyclase activity (Stubbs & Smith, 1984; Sastry, 1985).

Research in infant nutrition has demonstrated that during the last trimester of gestation the fetal brain accrues fatty acids of the n-6 and n-3 types (Clandinin et al., 1980a). These fatty acids may be derived from the placenta *in utero* with formation of major neural tissue requiring approximately 43 mg of n-6 and 22 mg of n-3 fatty acids per week (Clandinin et al., 1980a and 1980b; Clandinin et al., 1981). The accretion of essential fatty acids in neural tissues is predominately C20:4n-6 and C22:6n-3 (Clandinin et al., 1980b). It has also been estimated that requirements for n-6 and n-3 fatty acids in neuronal tissue synthesis can only be supplied from labile hepatic fatty acid reserves for 9 and 2.3 days, respectively, (Clandinin et al., 1981). The essential fatty acid reserves in the adipose tissue develops during the last trimester of fetal growth (Clandinin et al., 1981). Thus, the hepatic and adipose reserves cannot meet whole body needs for essential fatty acids and total fat if fetal development is interrupted by premature birth early in the third trimester.

Quantitative analysis of the composition of human milk from mothers giving birth to preterm infants (Clandinin et al., 1981) indicates that mothers' milk provides levels of C20:4n-6 and C22:6n-3 essential fatty acids approximating the predicted requirements at day 16 of life at oral intake levels of approximately 120 kcal / kg of body weight

(Clandinin et al., 1981). Long chain essential fatty acids are synthesized from C18:2n-6 or C18:3n-3; however, the amounts produced *in vivo* may be inadequate to support the accretion rates attained in breast-fed infants (Salem et al., 1996). Thus, it seems prudent to feed the preterm infant human milk or formulas with a fatty acid balance similar to human milk containing long chain polyenoic homologs of C18:2n-6 and C18:3n-3 (Clandinin et al., 1982).

Currently, infant formulas marketed in North America contain lC18:2n-6 and C18:3n-3 and are devoid of C20:4n-6 and C22:6n-3 (Clandinin et al., 1992a and 1992b). Therefore, infants fed these formulas must rely on *in vivo* elongation and desaturation of C18:2n-6 and C18:3n-3 to support a similar rate of accretion of C20:4n-6 and C22:6n-3 to that attained in breast-fed infants (Clandinin et al., 1981; Salem et al., 1996). It has been proposed (Clandinin et al., 1981; Clandinin et al., 1982) and recommended (European Society of Pediatric Gastroenterology and Nutrition, 1991; British Nutrition Foundation, 1992; Food and Agriculture Organization/World Health Organization, 1994; International Society for the Study of Fatty Acids and Lipids, 1994) that formulas fed to preterm infants be designed with a fatty acid balance similar to human milk containing C20:4n-6 and C22:6n-3. In the United Kingdom, Europe, South America, and Australia C20:4n-6 and C22:6n-3 have been added to preterm infant formulas using single-cell oils and in Europe using phospholipids.

In infants formulas, the question persists if preformed C22:6n-3 is needed or if providing more C18:3n-3 can be synthesized into C22:6n-3. In weanling rats, increasing dietary C18:3n-3 by decreasing C18:2n-6 to C18:3n-3 ratio from 7.3:1 to 4:1 increased the C22:6n-3 content in neuronal cell PE but not in other phospholipids from the cerebellum (Jumpsen et al., 1997). Results from other studies using whole brain (Woods et al., 1996) or subcellular fractions (Dyer & Greenwood, 1991) have also shown increases in C22:6n-3 content with increasing dietary C18:3n-3. However, none of these studies have investigated the effects of increasing C18:3n-3 on individual cell types from whole brain. Therefore, the present study utilized neonatal rat brain at two weeks of age, before the consumption of solid food, to test the hypothesis that increasing maternal dietary C18:3n-3 content from 1.6% (C18:2n-6 to C18:3n-3=21.6:1) to 17.5% (C18:2n-6 to C18:3n-3=1:1) of the total fatty acids will increase the C22:6n-3 content of neuronal cell phospholipids of rat pups. The results from this present study show that increasing maternal dietary C18:3n-3 by decreasing C18:2n-6 to C18:3n-3 ratio does not significantly increase the C22:6n-3 content in PC, PE, and PS of neuronal cell phospholipids of rat pups at two weeks of age.

B. MATERIALS AND METHODS

1. Animal Care

All animal procedures were approved by the University of Alberta Animal Ethics Committee. Sprague-Dawley rats were obtained from the University of Alberta vivarium. During breeding, three females and one male were housed together for a two-week mating period. Females were then moved to individual cages in a room maintained at 21°C with a 12 hr light and 12 hr dark cycle. Water and food were supplied *ad libitum.* Laboratory rodent diet, 5001 (PMI Feeds, Inc., St. Louis, MO) was fed to the rats when not receiving experimental diets. Rats were switched to experimental diet on the day of parturition. All litters were culled to twelve pups within 24 hr of parturition. Pups received only maternal milk. Pups were sacrificed at two weeks of age.

One entire litter of rat pups fed the same diet was sexed and weighed prior to decapitation. Excised brains were placed in ice-cold 0.32 mol/L sucrose. Six brains from the same sex were pooled per sample. Stomach contents of three rats from each litter were also removed and analyzed for fatty acid composition to reflect the composition of maternal milk. Three litters per diet treatment were used.

2. Diets

The basal diet fed meets all essential nutrient requirements and contained 20% (wt/wt) fat of varying C18:2n-6 and C18:3n-3 fatty acid composition (Clandinin & Yamashiro, 1980). Diet fats were formulated to approximate the fatty acid composition of an existing infant formula providing an C18:2n-6 to C18:3n-3 ratio of 7.3 to 1. This fat blend served as the control fat treatment. Three experimental diets were formulated by addition of various triglycerides to alter the fatty acid composition of this control fat formulation (Table 3.1). An C18:2n-6 to C18:3n-3 fatty acid ratio of 21.6 to 1 was obtained by addition of corn oil to the diet fat blend. The C18:2n-6 to C18:3n-3 fatty acid ratio of 4.4 to 1 and 1 to 1 was obtained by the addition of flaxseed oil. These diets were nutritionally adequate, providing for all known essential nutrient requirements [ingredient and concentration (g/kg diet), respectively]: fat 200; starch, 200; casein, 270; glucose, 207.65; nonnutritive fiber, 50; vitamin mix, 10; mineral mix, 50.85; L-methionine, 2.5; choline 2.75; and inositol, 6.25. The A.O.A.C. vitamin mix (Teklad Test Diets, Madison, WI) provided the following per kilogram of complete diet: 20,000 IU of vitamin A; 2,000 IU of vitamin D; 100 mg of vitamin E; 5 mg of menadione; 5 mg of thiamine-HCl; 8 mg of riboflavin; 40 mg of pyridoxine-HCl; 40 mg of niacin; 40 mg of pantothenic acid; 2,000 mg of choline, 100 mg of myoinositol; 100 mg of *p*-aminobenzoic acid; 0.4 mg of biotin; 2 mg of folic acid, and 30 mg of vitamin B_{12}; Bernhart Tomarelli mineral mix (General Biochemicals, Chargin Falls, OH) was modified to provide 77.5 mg of Mn^{2+} and 0.06 mg Se^{2+} per kilogram of complete diet. In order to minimize any changes in sample composition due to fatty acid oxidation, the diets were sealed under nitrogen and stored in a freezer at -30°C in darkness. Every day the required amount of diet was taken out, mixed, and placed in individual feed cups.

Table 3.1 Fatty Acid Composition of Experimental Diets Fed to Lactating Dams at Two Weeks of Age

	Diet C18:2n-6 to C18:3n-3 Ratio			
	21.6:1[1]	7.8:1[2]	4.4:1[3]	1:1[3]
Diet Fatty Acid Composition (% wt/wt)				
C12:0	6.4	8.4	9.4	6.0
C14:0	3.8	5.2	5.5	3.6
C16:0	12.2	14.0	13.9	11.4
C18:0	9.8	7.4	6.9	6.4
C18:1n-7+n-9	29.2	39.9	37.4	33.8
C18:2n-6	34.5	17.2	16.6	17.6
C18:3n-3	1.6	2.2	3.8	17.5
C20:4n-6	nd	nd	nd	nd
C22:6n-3	nd	nd	nd	nd

[1] C18:2n-6:C18:3n-3 of 21.6:1 was obtained by the addition of corn oil to the fat blend.

[2] C18:2n-6:C18:3n-3 of 7.8:1 diet approximates the fatty acid composition used in SMA® infant formula. Other diet blends were achieved by mixing corn oil (diet C18:2n-6:C18:3n-3 fatty acid ratio of 21.6:1) or flaxseed oil (diet C18:2n-6:C18:3n-3 fatty acid ratio of 1:1 or 4.4:1) into this basic fat blend.

[3] C18:2n-6:C18:3n-3 of 4.4:1 and 1:1 was obtained by the addition of flaxseed oil.

nd = not detected

3. Isolation of Neuronal Cells

Neuronal cells were isolated according to the method described by Sellinger & Azcurra (1974). Briefly, pooled brains were placed in beakers containing 7.5% (wt/vol.) polyvinylpyrrolidone and 10 mmol $CaCl_2$/L at pH 4.7 and 25°C. Brain tissue was minced and poured into a 20 mL plastic syringe, fitted with a reusable filter unit (Millipore, Swinnex disc holder, 25mm). The sample was pressed, three times each, through a series of combined nylon mesh filters. The final filtrate volume was adjusted, then layered on a two-step sucrose gradient of 1.0 mol/L and 1.75 mol/L. Gradients were centrifuged in a Beckman SW-28 rotor at 41,000 g for 30 min at 4°C.

Neuronal cell bodies were recovered in the pellet. Aliquots of cells were stained with methylene blue and examined for purity under a light microscope (Zeiss, 1600X;

Appendix 1; Hamberger & Svennerholm, 1971). Gel electrophoresis and immunoblotting were performed to ensure purity of neuronal cell fractions prepared by these procedures (Jumpsen et al., 1997). Proteins isolated from neuronal cells were compared by gel electrophoresis and immunoblotting to neurofilament protein standard (data not shown). Neuronal cells isolated contain only neurofilament proteins.

4. Lipid Analysis

The neuronal cell lipid was extracted by a modified Folch method (Folch et al., 1957). Separation of individual phospholipids was completed on silica gel thin-layer chromatography (TLC) H-plates (20x20 cm, Analtech, Newark, DE, USA). The plates were developed in a solvent system containing chloroform:methanol:triethylamine:1-propanol:0.25% (wt/vol.) KCl (30:9:18:25:6, by vol.) for approximately 90 min (Touchstone et al., 1980). TLC plates were air dried for 5 min and visualized with 0.1% (wt/vol.) anilino napthalene sulfonic acid in water.

Phospholipid fractions on the plate corresponding to standards were scraped into culture tubes. Fatty acid methyl esters were prepared with 14% (wt/wt) boron trifluoride in methanol following the method of Morrison & Smith (1964).

5. Fatty Acid Analysis

Fatty acid methyl esters were separated by automated gas-liquid chromatography, (Varian model 6000 GLC equipped with a Vista 654 data system and a Vista 8000 autosampler; Varian Instruments, Georgetown, ON) using a bonded fused silica BP20 capillary column (25 mm x 0.25 mm inside diameter) and quantitated using a flame ionization detector (Hargreaves & Clandinin, 1987). These conditions are capable of separating methyl esters of saturated, cis-monounsaturated, and cis-PUFA from 14 to 24 carbons in chain length. Quantitation and identification of peaks was based on relative retention times compared to known standards (PUFA 1 and 2, bacterial methyl ester mix-14; Supelco Canada, Mississauga, ON, Canada; Hargreaves & Clandinin, 1987).

6. Statistical Analysis

The effect of diet treatment and sex of rat pups on the fatty acid composition of neuronal cell phospholipid fractions was assessed by two-way analysis of variance (ANOVA) procedures using the SAS package, version 6.11 (SAS Institute Inc., 1988). Significant differences between diet treatments and sex were determined by a Duncan's multiple range test at a significance level of $p<0.05$ after a significant ANOVA (Steel & Torrie, 1960). Values are expressed as mean ± SEM for $n=6$. Two-way ANOVA procedures were performed on six diet treatments (including DHA and AA + DHA), however, in this chapter statistical analysis on four diet treatments were presented for the fatty acid composition of neuronal cell phospholipid fractions in the figures and tables.

C. RESULTS

1. Growth Characteristics

No significant differences were observed between males and females for body weight or total brain weight and the fatty acid composition of the individual phospholipid fractions. Thus, statistical analyses to test subsequent effects of diet treatments were combined for both sexes. Body and brain weight did not differ significantly between rat pups fed the four experimental diets. Final body weights were (mean ± SEM): 35.8 ± 0.9 g, 35.9 ± 1.0 g, 35.3 ± 0.8 g, and 35.6 ± 1.3 g for 21.6:1, 7.8:1, 4.4:1, and 1:1 diet treatments, respectively. Final brain weights were (mean ± SEM): 1.2 ± 0.1 g, 1.2 ± 0.1 g, 1.2 ± 0.1 g, and 1.1 ± 0.1 g for 21.6:1, 7.8:1, 4.4:1, and 1:1 diet treatments, respectively.

2. Purity of Neuronal Cell Preparations

The neuronal cell preparations contained only minor cross contamination (≈5%) from cell membrane fragments and microvessels as determined by microscopic examination. The presence of neurofilament in neuronal samples were previously verified by gel electrophoresis and immunoblotting (Jumpsen et al., 1997). These results indicate that the neuronal cell preparation is primarily neuronal cell bodies with attached extensions.

3. Fatty Acid Composition of Stomach Contents

The fatty acid composition of stomach contents of rat pups was analyzed. These analyses relate to the dams' milk composition (Nouvelot et al., 1983; Lien et al., 1994; Jumpsen et al., 1997). The increase in dietary C18:2n-6 or C18:3n-3 fed to the dams altered the stomach contents of the rat pups (Table 3.2) indicating that the range of dietary fat composition fed in the present experiment produced changes in the fat composition of dams' milk.

Table 3.2 The Content of Fatty Acids in the Stomach of Rat Pups at Two Weeks of Age[1]

	Diet C18:2n-6 to C18:3n-3 Ratio			
	21.6:1[2]	**7.8:1[3]**	**4.4:1[4]**	**1:1[4]**
Stomach Content of n-6 and n-3 fatty acid (% wt/wt)				
C10:0	7.9 ± 2.11^{b}	7.2 ± 0.78^{b}	8.9 ± 1.90^{a}	6.9 ± 2.23^{b}
C12:0	10.9 ± 1.12^{b}	15.1 ± 0.36^{a}	16.0 ± 2.32^{a}	10.8 ± 0.58^{c}
C14:0	7.3 ± 0.31^{c}	15.3 ± 0.48^{a}	9.8 ± 0.53^{b}	7.2 ± 0.30^{c}
C16:0	12.6 ± 0.94^{b}	17.7 ± 0.66^{a}	13.9 ± 1.35^{b}	12.9 ± 0.43^{b}
C18:0	4.4 ± 0.35^{a}	3.4 ± 0.13^{b}	4.0 ± 0.19^{b}	4.8 ± 0.21^{a}
C18:1n-7+n-9	28.2 ± 1.5^{a}	24.7 ± 0.69^{a}	28.0 ± 1.5^{a}	26.7 ± 0.99^{a}
C18:2n-6	24.7 ± 0.05^{a}	12.5 ± 0.07^{c}	10.4 ± 0.08^{d}	13.7 ± 0.07^{b}
C18:3n-3	1.1 ± 0.02^{d}	1.6 ± 0.03^{c}	2.1 ± 0.03^{b}	8.8 ± 0.13^{a}
C20:4n- 6	1.0 ± 0.07^{a}	0.5 ± 0.04^{b}	0.5 ± 0.02^{b}	0.5 ± 0.08^{b}
C22:6n-3	0.1 ± 0.02^{b}	0.1 ± 0.01^{b}	0.1 ± 0.02^{b}	0.2 ± 0.03^{a}
C18:2n-6:C18:3n-3	22.5	7.8	5.0	1.6

[1] Values are mean ± SEM with n=9 for each experimental diet. For each horizontal set of values, those that have the same superscript letters (a, b, c, or d) are not significantly different. Those that have different letters are statistically significantly different, at $p<0.05$.

[2] C18:2n-6:C18:3n-3 of 21.6:1 was obtained by the addition of corn oil to the fat blend.

[3] C18:2n-6:C18:3n-3 of 7.8:1 diet approximates the fatty acid composition used in SMA® infant formula. Other diet blends were achieved by mixing corn oil (diet C18:2n-6:C18:3n-3 fatty acid ratio of 21.6:1) or flaxseed oil (diet C18:2n-6:C18:3n-3 fatty acid ratio of 1:1 or 4.4:1) into this basic fat blend.

[4] C18:2n-6:C18:3n-3 of 4.4:1 and 1:1 was obtained by the addition of flaxseed oil.

4. Neuronal Cells Phospholipid Fatty Acid Composition

a) Phosphatidylcholine and Phosphatidylethanolamine

In brain, PC and PE are quantitatively the two most abundant phospholipids and constitute approximately 90% of total brain phospholipid (Green & Yavin, 1996). The major fatty acids observed in PC were C16:0, C18:0, and C18:1 (47-52%, 13-14%, 15-16% of total fatty acids, respectively). Feeding a maternal diet providing a ratio of C18:2n-6 to C18:3n-3 from 21.6:1 to 1:1 did not significantly alter the content of C20:4n-6 and C22:6n-3 in neuronal cell PC of rat pups ($p>0.05$) (Figure 3.1). In PE, C18:0, C20:4n-6, C22:6n-3, and C16:0 (23-28%, 16-20%, 16-22%, and 13-17% of total fatty acids, respectively) were the predominant fatty acids. Increasing C18:3n-3 in the maternal diet did not significantly alter the C20:4n-6 and C22:6n-3 content in neuronal cell PE of the rat pups ($p>0.05$) (Figure 3.1).

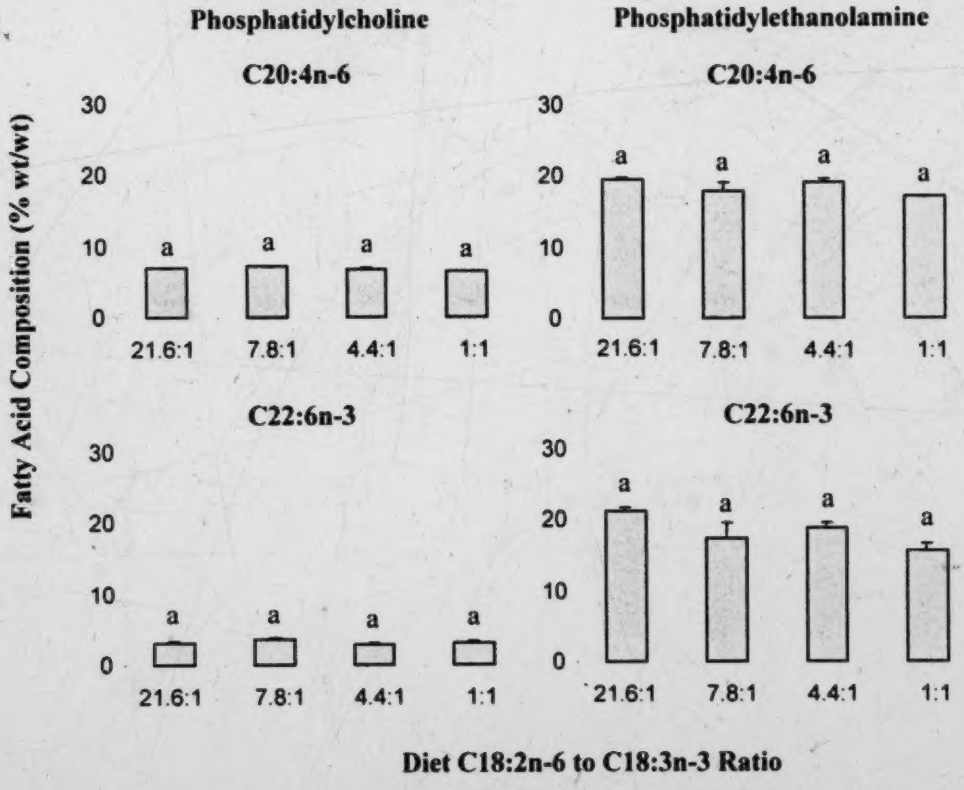

Figure 3.1 C20:4n-6 and C22:6n-3 Content in Neuronal Cell Phosphatidylcholine and Phosphatidylethanolamine

b) Phosphatidylserine and Phosphatidylinositol

Analysis of the fatty acid profile in neuronal cell PS demonstrated that C18:0 and C22:6n-3 (36-40% and 14-28% of the total fatty acid, respectively) were the major fatty acid. The large content of C22:6n-3 in PS of the rat pups was significantly decreased by increasing the maternal dietary levels of C18:3n-3 ($p<0.0001$). The maternal diet, providing a ratio of C18:2n-6 to C18:3n-3 ratio of 7.8 to 1, resulted in the highest level of C22:6n-3 in the rat pups (Table 3.3). The C16:0 content of PS increased from 7.2-20.1% of the total fatty acids when the C18:2n-6 to C18:3n-3 ratio was lowered from 7.8:1 to 1:1 (Table 3.3).

In neuronal cell PI, the major fatty acids were C18:0 and C20:4n-6 (28-39% and 19-35% of the total fatty acids, respectively). When increasing dietary levels of C18:3n-3 was provided in the maternal diet there was a significant increase in neuronal cell C22:6n-3 content of ≈11% from a dietary C18:2n-6:C18:3n-3 ratio of 7.8:1 to 4.4:1 ($p<0.05$) with a concomitant decrease in C20:4n-6 of ≈15.5% in PI in the rat pups (Table 3.4).

Table 3.3 Effect of Varying C18:2n-6 to C18:3n-3 Ratio on the Fatty Acid Composition of Neuronal Phosphatidylserine[1]

	Diet C18:2n-6 to C18:3n-3 Ratio			
	21.6:1	**7.8:1**	**4.4:1**	**1:1**
Fatty Acid (% wt/wt)				
C14:0	0.4 ± 0.0^{a}	0.3 ± 0.1^{a}	0.6 ± 0.1^{a}	0.7 ± 0.2^{a}
C16:0	13.4 ± 1.3^{b}	7.2 ± 0.5^{c}	15.0 ± 0.8^{b}	20.1 ± 2.7^{a}
C18:0	39.0 ± 1.3^{a}	36.9 ± 2.8^{a}	36.0 ± 1.7^{a}	38.8 ± 0.3^{a}
C18:1n-9	9.2 ± 0.3^{a}	9.0 ± 3.5^{a}	7.4 ± 1.8^{a}	10.5 ± 0.4^{a}
C18:1n-7	1.4 ± 0.1^{a}	0.5 ± 0.1^{b}	2.9 ± 1.4^{a}	1.8 ± 0.2^{a}
C18:2n-6	0.6 ± 0.0^{b}	0.4 ± 0.0^{c}	0.8 ± 0.0^{a}	0.8 ± 0.0^{a}
C20:4n-6	6.3 ± 0.1^{a}	6.2 ± 0.8^{a}	8.8 ± 1.6^{a}	6.1 ± 0.6^{a}
C22:4n-6	4.4 ± 0.2^{b}	5.4 ± 0.5^{a}	4.1 ± 0.3^{b}	3.0 ± 0.4^{c}
C22:5n-6	3.0 ± 0.1^{ab}	2.8 ± 0.1^{b}	1.7 ± 0.1^{ab}	1.3 ± 0.3^{a}
C18:3n-3	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.1 ± 0.0^{a}
C20:5n-3	0.1 ± 0.0^{b}	0.2 ± 0.1^{a}	0.1 ± 0.1^{b}	0.0 ± 0.0^{c}
C22:5n-3	0.2 ± 0.0^{b}	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}	0.4 ± 0.1^{a}
C22:6n-3	20.0 ± 1.1^{b}	28.0 ± 0.7^{a}	20.1 ± 1.1^{b}	14.1 ± 2.9^{c}

[1] Values are mean ± SEM with n=6 for each experimental diet. For each horizontal set of values, those that have the same superscript letters (a or b) are not significantly different. Those that have different letters are statistically significantly different, at $p<0.05$.

Table 3.4 Effect of Varying C18:2n-6 to C18:3n-3 Ratio on the Fatty Acid Composition of Neuronal Phosphatidylinositol[1]

	Diet C18:2n-6 to C18:3n-3 Ratio			
	21.6:1	**7.8:1**	**4.4:1**	**1:1**
Fatty Acid (%wt/wt)				
C14:0	0.9 ± 0.1^{a}	0.6 ± 0.1^{a}	0.4 ± 0.0^{a}	0.8 ± 0.1^{a}
C16:0	13.8 ± 1.3^{ab}	7.4 ± 1.7^{b}	12.5 ± 0.5^{ab}	15.2 ± 0.3^{a}
C18:0	28.1 ± 1.3^{c}	34.7 ± 0.5^{ab}	38.6 ± 1.9^{a}	31.0 ± 1.0^{bc}
C18:1n-9	8.1 ± 0.6^{b}	6.1 ± 0.7^{b}	1.8 ± 1.5^{c}	12.3 ± 2.1^{a}
C18:1n-7	1.8 ± 0.1^{ab}	1.4 ± 0.1^{bc}	0.4 ± 0.3^{c}	2.2 ± 0.1^{a}
C18:2n-6	0.8 ± 0.0^{b}	0.7 ± 0.1^{b}	0.8 ± 0.0^{b}	1.0 ± 0.0^{a}
C20:4n-6	26.5 ± 1.6^{b}	35.4 ± 2.3^{a}	19.9 ± 0.2^{c}	19.0 ± 1.4^{c}
C22:4n-6	3.1 ± 0.5^{a}	1.8 ± 0.1^{b}	3.9 ± 0.4^{a}	3.0 ± 0.1^{a}
C22:5n-6	1.3 ± 0.2^{a}	0.5 ± 0.1^{b}	1.0 ± 0.1^{ab}	0.8 ± 0.0^{b}
C18:3n-3	0.3 ± 0.3^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
C20:5n-3	0.4 ± 0.3^{a}	0.1 ± 0.0^{a}	0.2 ± 0.1^{a}	0.1 ± 0.0^{b}
C22:5n-3	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.3 ± 0.0^{a}	0.4 ± 0.0^{a}
C22:6n-3	11.1 ± 1.6^{b}	6.2 ± 0.3^{c}	17.5 ± 2.0^{a}	10.7 ± 0.5^{b}

[1] Values are mean ± SEM with n=6 for each experimental diet. For each horizontal set of values, those that have the same superscript letters (a, b, or c) are not significantly different. Those that have different letters are statistically significantly different, at $p<0.05$.

D. DISCUSSION

The present study was initiated to determine the effects of increasing maternal dietary C18:3n-3 content by decreasing the C18:2n-6 to C18:3n-3 ratio from 21.6:1 to 1:1 on the C22:6n-3 content in neuronal cells of rat pups at two weeks of age. The results demonstrate that increasing maternal dietary C18:3n-3 content does not significantly increase the C22:6n-3 content of neuronal cell PC, PE, and PS of rat pups at a stage of brain development when C22:6n-3 is needed for rapid neural plasma membrane synthesis.

The reason for the similar C20:4n-6 and C22:6n-3 content in PC and PE of neuronal cells between the four experimental diets may be due to the fact that desaturase activity is age-related (Bordoni et al., 1988; Hrelia et al., 1989; Bourre et al., 1990; Ulmann et al., 1991) and that at two-weeks of age activity may be limited. Bourre et al. (1990) demonstrated in rats that delta-6 desaturase activity, a rate-limiting step in C20:4n-6 and C22:6n-3 synthesis (Stoffel, 1961; Holloway et al., 1963; Brenner, 1971) varies during the first 21 days following gestation. Therefore, if delta-6 desaturase activity is low at two-weeks of age, the amount of C18:2n-6 and C18:3n-3 added in the diet would not have any significant effect on increasing the C20:4n-6 and C22:6n-3 content of membrane phospholipids.

The significant decrease in C22:6n-3 content in PS with increasing dietary C18:3n-3 may be attributed to different PS molecular species being produced by deacylation and reacylation processes (Lands, 1960).

PI represents approximately 4% of the total brain phospholipids (Green & Yavin, 1996). The deacylation of C20:4n-6 from PI followed by reacylation of PI with C22:6n-3 from other phospholipids such as PS could account for the decrease in C20:4n-6 and increase in C22:6n-3 content in PI when the C18:2n-6 to C18:3n-3 ratio was decreased from 7.8:1 to 1:1 (Table 3.4).

PS and PI although minor phospholipids in brain membranes are of special interest because both are involved in cellular functions (Berridge, 1984). PS is responsible for the activation of several protein kinase C isoforms (Epand & Lester, 1990) while PI plays an important role in signal transduction (Hokin, 1985) and in production of eicosanoids (Wood, 1986). Moreover, the fatty acyl composition of PS and PI has been demonstrated to be one of the regulatory functions in enzyme activation (Bolen & Sando, 1991). Therefore, the changes observed in PS in C22:6n-3 content and PI C20:4n-6 and C22:6n-3 content, while small on the basis of total brain content, could have functional implications.

In vivo studies have suggested that it is more effective to supply a dietary source of preformed C22:6n-3 to maintain the C22:6n-3 level in membrane phospholipids, rather than increasing the dietary content of C18:3n-3 (Sinclair, 1975; Anderson et al., 1990). The results from the present study appear to support these findings since increasing the dietary C18:3n-3 content by ≈11 fold (Table 3.1) did not significantly increase the C22:6n-3 content in neuronal cell PC, PE, and PS (Figure 3.1 and Table 3.3).

The findings of this study may have important implications for neonatal feeding. If the present findings in neonatal rats are extrapolated to infants, it appears that increasing the C18:3n-3 content by decreasing the C18:2n-6 to C18:3n-3 ratio in preterm infant formulas will not stimulate an increase in levels of C22:6n-3 in the early neonatal period.

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CHAPTER IV. HIGH DIETARY C18:3N-3 INCREASES THE C18:3N-3 BUT NOT THE C22:6N-3 CONTENT IN THE WHOLE BODY, BRAIN, SKIN, EPIDIDYMAL FAT PADS AND MUSCLES OF SUCKLING RAT PUPS

A. INTRODUCTION

Research in infant nutrition has demonstrated that during the last trimester of gestation the fetal brain accrues fatty acids of the n-6 and n-3 types (Clandinin et al., 1980b). These fatty acids may be derived from the placenta *in utero* (Clandinin et al., 1980a and 1980b; Clandinin et al., 1981). The hepatic and adipose reserves cannot meet whole body needs for essential fatty acids and total fat if fetal development is interrupted by premature birth early in the third trimester (Clandinin et al., 1981). Mothers' milk provides C20:4n-6 and C22:6n-3 approximating the predicted requirements at day 16 of life at oral intake (Clandinin et al., 1981). Long chain essential fatty acids are synthesized from C18:2n-6 or C18:3n-3; however, the amounts produced *in vivo* may be inadequate to support the accretion rates attained in breast-fed infants (Demmelmair et al., 1995; Carnielli et al., 1996; Salem et al., 1996; Sauerwald et al., 1996; Sauerwald et al., 1997). Deficiencies of dietary C22:6n-3 during perinatal development results in poor C22:6n-3 accretion in brain and retina, subsequently leading to altered neurological and visual function in animals (Bourre et al., 1989; Connor et al., 1990). Thus, it seems prudent to feed the preterm infant human milk or formulas with a fatty acid balance similar to human milk containing long chain polyenoic homologs of C18:2n-6 and C18:3n-3 (Clandinin et al., 1982).

Current infant formulas in North America contain C18:2n-6 and C18:3n-3 but are devoid of C20:4n-6 and C22:6n-3 (Clandinin et al., 1992a and 1992b). Infants fed these formulas must rely on *in vivo* elongation and desaturation of C18:2n-6 and C18:3n-3 to support a similar rate of accretion of C20:4n-6 and C22:6n-3 to that attained in breast-fed infants. Addition of C20:4n-6 and C22:6n-3 to preterm infant formulas to optimize brain development has been recommended (Clandinin et al., 1981; Clandinin et al., 1982; European Society of Pediatric Gastroenterology and Nutrition, 1991; British Nutrition Foundation, 1992; International Society for the Study of Fatty Acids and Lipids, 1994; FAO/WHO, 1994). Infant formulas supplemented with C20:4n-6 and C22:6n-3 produce a clear dose response in the content of C20:4n-6 and C22:6n-3 in erythrocyte total plasma membrane phospholipids with 0.6% C20:4n-6 and 0.4% C22:6n-3 in the formula fat providing sufficient amounts of these fatty acids to achieve a fatty acid composition of C20:4n-6 and C22:6n-3 similar to that of infants fed human milk (Clandinin et al., 1997). Thus, in the United Kingdom, Europe, South America, Australia, and the Middle East C20:4n-6 and C22:6n-3 have been added to infant formulas.

The question of whether preformed C22:6n-3 is needed or if providing more C18:3n-3 can enable synthesis of C22:6n-3 persists. In human adults that it is more effective to supply a dietary source of preformed C22:6n-3 from fish oil to increase the C22:6n-3 content in plasma phospholipids than C18:3n-3 from flaxseed oil (Layne et al., 1996). Increasing maternal dietary C18:3n-3 by decreasing the diet C18:2n-6:C18:3n-3 from 7.3:1 to 4:1 was not as effective as preformed C22:6n-3 at raising the C22:6n-3 content of neuronal cell phospholipids of weanling rats (Jumpsen et al., 1997a and 1997b). Increasing maternal dietary C18:3n-3 content does not significantly increase the C22:6n-3 content in PC, PE, or PS of neuronal cell phospholipids of rat pups at two-weeks of age (**CHAPTER III**). These observations beg the question if C18:3n-3 does not give rise to significant quantities of C22:6n-3 then what is the metabolic fate of high intakes of C18:3n-3?

The purpose of this study was to determine if the dietary C18:3n-3 consumed could increase the C18:3n-3 and C22:6n-3 content of other whole body tissue lipids. It is hypothesized that increasing maternal dietary C18:3n-3 by decreasing the C18:2n-6:C18:3n-3 ratio will increase the C18:3n-3 and C22:6n-3 content in the whole body, liver, skin (epidermis, dermis, and subcutaneous tissue), epididymal fat pads, and muscles (front and back legs) in two-week-old rat pups.

B. MATERIALS AND METHODS

1. Animals and Diets

Breeding of Sprague-Dawley rats have been described in **Chapter III**. Pups were sacrificed at two weeks of age. One entire litter of rat pups fed the same experimental diet was sexed and weighed prior to decapitation. Rat pups randomly chosen for whole body lipid analysis were frozen in liquid nitrogen. The brains, livers, skin (epidermis, dermis, and subcutaneous tissue), epididymal fat pads, and muscles (front and back legs) were quickly removed and rinsed with ice-cold physiological saline, blotted dry, weighed, and frozen in liquid nitrogen. Stomach contents of three rats from each litter were also removed and analyzed for fatty acid composition to reflect the composition of maternal milk. All tissue samples were stored under nitrogen and kept in a -80°C freezer until analysis. Analysis of whole organs was performed on at least three individual rat pups from each of three different litters per diet treatment.

The basal diet fed to the dams contained 20% (wt/wt) fat varying in C18:2n-6 and C18:3n-3 fat composition. Two experimental diets were formulated by addition of various triglycerides from vegetable oils to alter the fatty acid C18:2n-6 : C18:3n-3 ratio (Table 4.1). An C18:2n-6 to C18:3n-3 fatty acid ratio of 24.7 to 1 (low C18:3n-3) was obtained by addition of corn oil to the diet fat blend. The C18:2n-6 to C18:3n-3 fatty acid ratio of 1 to 1 (high C18:3n-3) was obtained by the addition of flaxseed oil. The low and high C18:3n-3 fatty acid diet was nutritionally adequate, providing for all known essential nutrient requirements as described earlier (Clandinin & Yamashiro, 1980). To minimize any changes in sample composition due to fatty acid oxidation, the diets were sealed under nitrogen and stored in a freezer at -30°C in darkness. Every day the required amount of diet was taken out, mixed, and placed in individual feed cups.

2. Lipid Extraction and fatty acid analysis

Total lipids were extracted from aliquots of tissue homogenate (Folch et al., 1957). The extracted lipids were evaporated under nitrogen and weighed to determine the total fat content of the tissues. The tissue lipids were saponified by a 0.5N KOH in 95% methanol solution and heated at 100°C for 1 hr. Fatty acid methyl esters were prepared using BF_3 / methanol reagent (Morrison & Smith, 1964).

Fatty acid methyl esters were analyzed by automated gas-liquid chromatography as described in **Chapter III**.

Table 4.1 Fatty Acid Composition of Experimental Diets Fed to Lactating Dams and Stomach Content of Rat Pups of Two Weeks of Age^a

	Diet		Stomach Content	
	Low C18:3n-3	High C18:3n-3	Low C18:3n-3	High C18:3n-3
Fatty Acid (% wt/wt)				
C12:0	15.3	7.90	0.2 ± 0.0^a	0.1 ± 0.02^a
C14:0	6.8	3.1	20.2 ± 1.5^a	16.11 ± 1.4^a
C16:0	13.1	9.0	21.1 ± 0.3^a	18.5 ± 0.8^b
C18:0	4.7	3.7	4.0 ± 0.2^a	3.3 ± 0.2^b
C18:1n-7+n-9	26.4	27.2	26.7 ± 0.9^a	26.2 ± 0.7^a
C18:2n-6	27.2	20.4	25.4 ± 0.4^a	19.3 ± 0.4^b
C18:3n-3	1.1	20.1	1.1 ± 0.2^b	14.9 ± 1.1^a
C20:4n-6	nd	nd	0.7 ± 0.1^a	0.4 ± 0.1^a
C22:6n-3	nd	nd	0.04 ± 0.01^a	0.12 ± 0.0^a
Σ Sat^b	43.4	30.3	46.1 ± 1.3^a	38.7 ± 2.1^b
Σ Mono^c	28.2	29.2	26.7 ± 0.9^a	26.2 ± 0.7^a
Σ N-6^d	27.2	20.4	26.1 ± 0.5^a	19.7 ± 0.4^b
Σ N-3^e	1.3	20.1	1.2 ± 0.2^b	15.4 ± 1.2^a
C18:2n-6 to C18:3n-3	24.7:1	1.0:1	23.1:1	1.3:1

nd=not detected^a
Values represent mean ± SEM for n=9 rat pups (5 male and 4 female) for each experimental diet. Values without a common superscript are significantly different at $P<0.05$.
[b] Σ Sat, sum of saturated fatty acids
[c] Σ Mono, sum of monounsaturated fatty acids
[d] Σ N-6, sum of N-6 fatty acids
[e] Σ N-3, sum of N-3 fatty acids

3. Statistical Analysis

The effect of diet treatment and sex of rat pups on the lipid content and fatty acid composition of whole body and tissues lipids was assessed by one-way analysis of variance (ANOVA) procedures using the SAS package, version 6.11 (SAS Institute Inc., 1988). Significant differences between diet treatments and sex were determined by a

Duncan's multiple range test at a significance level of $p<0.05$ after a significant ANOVA (Steel & Torrie, 1960). Values are expressed as mean ± SEM for n=9 rat pups.

C. RESULTS

1. Fatty Acid Composition of Stomach Contents.

The stomach content of rat pups was analyzed for the fatty acid composition. These analyses reflected dams' milk composition (Nouvelot et al., 1983; Yonekubo et al., 1993; Lien et al., 1994; Jumpsen et al., 1997a and 1997b; Wainwright et al., 1997). The increase in dietary C18:3n-3 fed to the dams increased the C18:3n-3 in the stomach contents of the rat pups (Table 4.1) indicating that the range of dietary fat composition fed in the present experiment produced changes in the fat composition of the dams' milk as predicted.

2. Whole Body and Tissue Weights and Lipid Content

Whole body and tissue weights, lipid content, and fatty acid composition of the tissues were not significantly different for male (n=5) and female (n=4) rat pups (data not shown), hence in statistical analyses to test subsequent effects of diet treatments were combined for both sexes. The whole body and tissue weights were not significantly different among the two experimental diet treatments (Table 4.2), indicating that the body and tissue growth in the two-week-old rat pups is not different between low and high C18:3n-3 fatty acid diets. The lipid content of whole body and tissues was not significantly different in rat pups fed either the low or high C18:3n-3 fatty acid diet (Table 4.2). Differences in fertility were not observed between dietary treatments.

Table 4.2 Whole Body, Tissue Weights, and Lipid Content of Rat Pups at Two Weeks of Age[a]

	Weight (g)		% Lipid	
	Low C18:3n-3	High C18:3n-3	Low C18:3n-3	High C18:3n-3
Whole Body	31.8 ± 1.0^{a}	32.0 ± 1.2^{a}	13.6 ± 1.0^{a}	13.4 ± 0.8^{a}
Brain	1.15 ± 0.0^{a}	1.12 ± 0.1^{a}	4.40 ± 0.4^{a}	4.20 ± 0.3^{a}
Liver	0.66 ± 0.1^{a}	0.67 ± 0.1^{a}	6.90 ± 0.7^{a}	6.90 ± 0.8^{a}
Skin	9.00 ± 1.0^{a}	8.95 ± 1.0^{a}	24.0 ± 1.3^{a}	25.0 ± 1.1^{a}
Epididymal Fat Pads	0.05 ± 0.0^{a}	0.05 ± 0.0^{a}	nd	nd
Muscles	1.00 ± 0.0^{a}	0.91 ± 0.1^{a}	1.70 ± 0.0^{a}	1.60 ± 0.0^{a}

nd = not determined

[a] Values represent mean ± SEM for n=9 rat pups (5 male and 4 female rat pups except foe epididymal fat pads in which 9 male rat pups were used) for each experimental diet. Values without a common superscript are significantly different at P<0.05.

a) Brain

The brain of two-week-old rat pups contained small amounts of C18:2n-6 and C18:3n-3. The C18:2n-6 content of brain was significantly increased in animals fed the low compared to a high C18:3n-3 fatty acid diet (Table 4.3). The C18:3n-3 content of brain was significantly increased in rat pups fed the high compared to the low C18:3n-3 fatty acid diets (Table 4.3). C20:4n-6 and C22:6n-3 were the predominant n-6 and n-3 fatty acids in brain of rat pups fed low or high C18:3n-3 fatty acid diet, respectively. The C20:4n-6 content of brain was significantly increased in rat pups fed the low vs. high C18:3n-3 fatty acid diet (Table 4.3). The C22:6n-3 content of brain was not significantly different in the animals fed the high compared to the low C18:3n-3 fatty acid diets (Table 4.3). The C20:5n-3 content of brain was not significantly different between rat pups fed low vs. high C18:3n-3 fatty acid diet (Table 4.3). However, the C22:5n-3 content of brain was significantly different between rat pups fed low vs. high C18:3n-3 fatty acid diet (Table 4.3).

b) Liver

The partial fatty acid composition of liver from two-week-old rat pups is shown (Table 4.3). High content of C18:2n-6 and C20:4n-6 was observed in the liver of rat pups fed either a low or high C18:3n-3 fatty acid diet. The C18:3n-3 and C22:6n-3 content of liver was considerably lower than the C18:2n-6 and C20:4n-6 content for animals fed either the low or high C18:3n-3 diet. Significant differences were observed in the C18:2n-6 content of liver between rat pups fed low vs. high C18:3n-3 fatty acid diet. The content of C18:3n-3 in liver was approximately 3% in animals fed the high C18:3n-3 fatty acid diet and was significantly different when compared to animals fed the low C18:3n-3 fatty acid diet. The C20:4n-6 content of liver did not differ between rat pups fed low vs. high C18:3n-3 fatty acid diet (Table 4.3) whereas, the C22:6n-3 content of liver was significantly increased in animals fed high vs. low C18:3n-3 fatty acid diet (Table 4.3). The C20:5n-3 and C22:5n-3 content of liver was significantly different between rat pups fed low vs. high C18:3n-3 fatty acid diet (Table 4.3).

Table 4.3 Effect of Low and High C18:3n-3 Diet on the Essential Fatty Acid Composition of Whole Body and Tissue Lipids [a]

Fatty Acid (% wt/wt)	Whole Body		Brain		Liver		Skin		Epididymal Fat Pad		Muscles	
	Diet C18:3n-3 Content											
Diet 18:3n-3 Content :	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
C18:2n-6	$24.5^{a}\pm0.4$	$19.3^{b}\pm0.3$	$2.8^{a}\pm0.5$	$1.7^{b}\pm0.0$	$16.3^{a}\pm0.2$	$13.0^{b}\pm0.4$	$21.6^{a}\pm1.1$	$15.5^{b}\pm0.5$	$19.9^{a}\pm0.6$	$18.8^{a}\pm0.6$	$20.8^{a}\pm1.8$	$15.9^{b}\pm0.7$
C18:3n-3	$0.9^{b}\pm0.0$	$14.7^{a}\pm0.4$	$0.1^{b}\pm0.0$	$0.5^{a}\pm0.0$	$0.2^{b}\pm0.1$	$3.3^{a}\pm0.3$	$0.7^{b}\pm0.1$	$11.6^{a}\pm0.4$	$1.8^{b}\pm0.7$	$13.3^{a}\pm0.7$	$1.6^{b}\pm1.0$	$10.7^{b}\pm0.3$
C20:4n-6	$1.7^{a}\pm0.1$	$1.0^{b}\pm0.1$	$12.0^{a}\pm0.3$	$10.0^{b}\pm0.2$	$15.0^{a}\pm2.1$	$12.4^{a}\pm1.0$	$0.8^{a}\pm0.2$	$0.6^{a}\pm0.1$	$1.1^{a}\pm0.1$	$0.9^{a}\pm0.1$	$1.7^{a}\pm0.3$	$0.9^{b}\pm0.3$
C20:5n-3	$0.0^{b}\pm0.0$	$0.7^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.1^{a}\pm0.0$	$0.1^{b}\pm0.0$	$1.5^{a}\pm0.1$	$0.0^{b}\pm0.0$	$0.5^{a}\pm0.0$	$0.1^{b}\pm0.0$	$0.4^{a}\pm0.1$	$0.1^{b}\pm0.1$	$0.6^{a}\pm0.1$
C22:5n-3	$0.1^{b}\pm0.0$	$0.7^{a}\pm0.0$	$0.1^{b}\pm0.0$	$0.5^{a}\pm0.0$	$0.4^{b}\pm1.3$	$1.3^{a}\pm0.1$	$0.0^{b}\pm0.0$	$0.4^{a}\pm0.0$	$0.1^{b}\pm0.0$	$0.3^{a}\pm0.1$	$0.0^{a}\pm0.0$	$0.2^{a}\pm0.1$
C22:6n-3	$0.3^{a}\pm0.1$	$0.5^{a}\pm0.1$	$6.5^{a}\pm0.3$	$7.1^{a}\pm0.6$	$3.5^{b}\pm0.8$	$6.1^{a}\pm0.6$	$0.2^{a}\pm0.1$	$0.4^{a}\pm0.0$	$0.2^{a}\pm0.1$	$0.4^{a}\pm0.1$	$0.2^{a}\pm0.1$	$0.3^{a}\pm0.1$

[a] Values represent mean ± SEM for n=9 rat pups (5 male and 4 female rat pups except for epididymal fat pads in which 9 male rat pups were used) for each experimental diet. Values without a common superscript for each fatty acid are significantly different at $P<0.05$.

c) Skin (Epidermis, Dermis, and Subcutaneous Tissue)

The fatty acid composition of skin (epidermis, dermis, and subcutaneous tissue; Table 4.3) shows that C18:2n-6 plus C20:4n-6 were major fatty acids of skin comprising approximately 23 and 17% for animals fed the low and high C18:3n-3 fatty acid diet, respectively. The C18:2n-6 content in skin was significantly higher in rat pups fed low compared to a high C18:3n-3 fatty acid diet. However, there was no significant difference in C20:4n-6 content of skin among the two diet groups. Similar to liver, the C18:3n-3 content of skin was significantly increased by high maternal dietary C18:3n-3 content (Table 4.3). The C22:6n-3 content of skin, however, was not statistically different in animals fed either the low or high C18:3n-3 diet (Table 4.3). The C20:5n-3 and C22:5n-3 content of skin was significantly different between rat pups fed low vs. high C18:3n-3 fatty acid diet (Table 4.3).

d) Epididymal Fat Pads

Similar to skin and liver, the C18:2n-6 content of epididymal fat pads from male rat pups was high containing approximately 19-20% of the total fatty acids (Table 4.3). The C18:2n-6 content in epididymal fat pads was not significantly affected by the two dietary fat treatments. However, unlike C18:2n-6, the C18:3n-3 content of this tissue was significantly increased by the dietary C18:3n-3 content (Table 4.3). The content of C18:3n-3 in epididymal fat pads was approximately 1.8 and 13.3% for animals fed either low or high C18:3n-3 fatty acid diet, respectively. C20:4n-6 and C22:6n-3 content in epididymal fat pads was not significantly different between animals fed the two diet groups (Table 4.3). The C20:5n-3 and C22:5n-3 content of epididymal fat pads was significantly different between rat pups fed low vs. high C18:3n-3 diet (Table 4.3).

e) Muscles (Front and Back Legs)

The fatty acid composition of muscle (front and back legs) from two-week-old rat pups is shown (Table 4.3). The C18:2n-6 content in muscles was significantly different between rat pups fed low vs. high C18:3n-3 fatty acid diet. The C18:2n-6 content in muscles of rat pups fed low or high C18:3n-3 fatty acid diet was approximately 21 and 16%, respectively. The C18:3n-3 content of muscles was significantly different between rat pups fed low vs. high C18:3n-3 fatty acid diet (1.6 vs. 10.7%, respectively). The C20:4n-6 content in muscles of rat pups was significantly higher in the low compared to the high C18:3n-3 fatty acid diet (1.7 vs. 0.9%, respectively). No significant differences were observed in the C22:6n-3 content of muscles in the two diet groups. The C20:5n-3 but not the C22:5n-3 content of muscles was significantly different between rat pups fed low vs. high C18:3n-3 fatty acid diet (Table 4.3).

f) Tissue C18:3n-3 Content in Whole Body

Feeding a low C18:3n-3 fatty acid diet to the dams significantly increased the C18:2n-6 content of whole body lipids of the rat pups compared to the high C18:3n-3 fatty acid diet (Table 4.3). Raising the C18:3n-3 content from a low to a high C18:3n-3 fatty acid diet significantly increased the C18:3n-3 content of whole body lipids (Table 4.3). The C20:4n-6 content of whole body lipids was significantly different between rat pups fed low or high C18:3n-3 fatty acid diet. However, the C22:6n-3 content of whole body lipids was not significantly different between rat pups fed the low vs. high C18:3n-3 fatty acid diet (Table 4.3). The C20:5n-3 and C22:5n-3 content of whole body was significantly different between rat pups fed low vs. high C18:3n-3 fatty acid diet (Table 4.3). Based on the lipid and C18:3n-3, C20:5n-3, C22:5n-3, and C22:6n-3 content, as well as, the weight of the tissues examined in the present study, the C18:3n-3, C20:5n-3, C22:5n-3, and C22:6n-3 content in whole body of rat pups fed either low or high C18:3n-3 fatty acid diet was calculated to be approximately 39 and 630 mg, 0 and 30 mg, 4.3 and 30 mg, 12.9 and 21.4 mg, respectively (data not shown). The other tissues examined in this study including brain, liver, epididymal fat pads, and muscles (front and back legs) from two-week-old rat pups fed either low or high C18:3n-3 fatty acid diet contained small amounts of C18:3n-3, C20:5n-3, and C22:5n-3 (data not shown). Interestingly, the skin (epidermis, dermis, and subcutaneous tissue) of rat pups fed either low or high C18:3n-3 fatty acid diet contained a significant amount of C18:3n-3. The total C18:3n-3 content in skin for the low and high C18:3n-3 fatty acid diet was approximately 15 and 260 mg, respectively (data not shown).

D. DISCUSSION

The results from the present study demonstrate that increasing maternal dietary C18:3n-3 significantly increases the C18:3n-3 but not the C22:6n-3 content of whole body, brain, skin (epidermis, dermis, and subcutaneous tissue), epididymal fat pads, and muscles (front and back legs) in two-week-old rat pups. Two-week-old rat pups were used in the present study because at this age very active brain growth occurs with rapid accretion of PUFAs, especially C22:6n-3, for brain membrane synthesis (Sinclair & Crawford, 1972; Dobbing & Sands, 1979). Therefore, the demand for C22:6n-3 in two-week-old rat pups for postnatal brain growth and development is high and must be provided by the maternal diet.

It is well known that the fatty acid composition of tissues can be readily modified by dietary fat [reviewed by Clandinin et al. (1985 & 1991)]. The significant differences observed in whole body and tissue C18:2n-6 and C18:3n-3 content between rat pups fed low vs. high C18:3n-3 fatty acid diet were largely a reflection of the dietary fatty acid composition (Field et al., 1985; Bourre et al., 1990; Lin & Connor, 1990; Srinivasarao et al., 1997).

The content of C22:6n-3 was not significantly increased in whole body, brain, skin (epidermis, dermis, and subcutaneous tissue), epididymal fat pads, and muscles (front and back legs) when rat pups were fed the high C18:3n-3 diet. This could be because desaturase activity is age-related and that at two weeks of age desaturase activity may be limited. In this regard, Bourre et al. (1990) demonstrated in rats that delta-6-desaturase activity, a rate-limiting step in C20:4n-6 and C22:6n-3 synthesis varies during the first twenty-one days following gestation. Therefore, if delta-6-desaturase activity is low at two weeks of age, the amount of C18:3n-3 added in the diet would not have any significant effect on increasing the C22:6n-3 content of these tissues. The significant increase in C20:5n-3 (except brain) and C22:5n-3 (except muscles) but not C22:6n-3 content of tissues examined in this study when rat pups were fed the high compared with low C18:3n-3 diet shows that C18:3n-3 is metabolized to long-chain n-3 metabolites but that there is a limit on the conversion of C20:5n-3 and C22:5n-3 to C22:6n-3.

The milk (stomach contents) provided to the rat pups by the dam during the two-week feeding period provide some preformed C22:6n-3 (Table 4.1). It is possible that the preformed C22:6n-3 (0.1%) present in the dams' milk fed the high C18:3n-3 fatty acid diet (Table 4.1) is sufficient to significantly increase the content of C22:6n-3 observed in liver of rat pups fed the high C18:3n-3 fatty acid diet (Table 4.3) without the need for additional synthesis of C22:6n-3 from dietary C18:3n-3. With exception of brain, increasing dietary C18:3n-3 by reducing the C18:2n-6:C18:3n-3 ratio from 24.7:1 to 1.0:1 did not show any competitive effect of reduced C20:4n-6 and increased C22:6n-3 incorporation into whole body or tissue lipids. This would suggest that either desaturase activity is low or that a lower C18:2n-6:C18:3n-3 ratio than that used in the present study may be required to reduce the C20:4n-6 and increase the C22:6n-3 content in the tissues examined (Jumpsen et al., 1997a).

Quantitative analysis of the C18:3n-3 content in rat pup tissues examined in the present study showed that significant amount of C18:3n-3 was incorporated into whole body lipids. The C18:3n-3 content of rat pup whole body lipids was significantly greater in rat pups fed the high compared to the low C18:3n-3 diet (approximately 630 vs. 39 mg, respectively). The skin of rat pups fed low or high C18:3n-3 fatty acid diet contained significant quantities of C18:3n-3 (approximately 39 and 41% of the total C18:3n-3 content in whole body, respectively). The high amounts of C18:3n-3 found in skin was stored as part of the fatty acid component of triglycerides in the subcutaneous fat (data not shown). Thus, it appears that the skin including epidermis, dermis, and subcutaneous tissue, is a major deposition site for C18:3n-3 in two-week-old rat pups.

The saturated fatty acid content in whole body, skin, and epididymal fat pads was significantly decreased ($p<0.05$) when the pups were fed maternal milk from the dams fed the high C18:3n-3 diet (data not shown). This decrease in the saturated fatty acid content of tissues was due to the substantial increase in the content of C18:3n-3 in these tissues by the high C18:3n-3 diet.

In conclusion, the results from the present study demonstrate that increasing maternal dietary C18:3n-3 content from 1.1 to 20.1% of the total dietary fatty acids significantly increases the C18:3n-3 but not the C22:6n-3 content in most tissues. If the present findings from this study are extrapolated to neonates, it appears that increasing the dietary C18:3n-3 content of the neonate's feed will significantly increase the C18:3n-3 but not the C22:6n-3 content of neonatal tissues.

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CHAPTER V. DIETARY C22:6N-3 IS MORE EFFECTIVE THAN LOW OR HIGH LEVELS OF C18:3N-3 IN INCREASING THE C22:6N-3 CONTENT IN PHOSPHOLIPIDS OF NEURONAL AND GLIAL CELLS FROM WHOLE BRAIN IN NEONATAL RAT

A. INTRODUCTION

C20:4n-6 and C22:6n-3 are the most abundant PUFAs in phospholipids of the central nervous system (O'Brien & Sampson, 1965; Sun & Horrocks, 1968) and can be synthesized in animal tissues by desaturation and elongation of their dietary precursors, C18:2n-6 and C18:3n-3 (Dhopeshwarkar & Subramanian, 1976). C20:4n-6 and C22:6n-3 are found in high concentrations in brain SPM (Cotman et al., 1969; Breckenridge et al., 1972; Sun & Sun, 1974; Foot et al., 1982) and in photoreceptor cells (Anderson et al., 1974). C20:4n-6 plays an important role as a precursor of biologically active molecules like prostanoids, leukotrienes, and other lipoxygenase products (reviewed by Kinsella et al., 1990). C20:4n-6 and C22:6n-3 are involved in providing a specific environment within the phospholipid bilayer that influence important membrane functions, such as, ion or solute transport, receptor activity, and adenylate cyclase activity (reviewed by Stubbs & Smith, 1984; Sastry, 1985).

C20:4n-6 and C22:6n-3 accumulate rapidly in the brain during the fetal and early postnatal periods, depending on the species. In rats, C22:6n-3 accumulates during the embryonic period and first three postnatal weeks of life (Kishimoto et al., 1965; Sinclair & Crawford, 1972; Green et al., 1999). In humans, accretion of C20:4n-6 and C22:6n-3 takes place during the last trimester and first 6 – 10 months after birth (Clandinin et al., 1980a & 1980b). The rapid accumulation of C20:4n-6 and C22:6n-3 in rat and human brain suggests that these fatty acids may be essential for neural and visual development. Manipulation of brain C22:6n-3 content by C22:6n-3 deprivation has been attempted to determine some functions of C22:6n-3 in the central nervous system. In this regard, rats fed diets deficient in C18:3n-3 but with adequate C18:2n-6 had lower levels of C22:6n-3 in brain and retina compared to rats fed C18:3n-3 (Bourre et al., 1989) and have delayed electrophysiological responses in the retina (Bourre et al., 1989) together with poorer performance in behavioral tests of learning, memory, and habituation (Lamptey &

Walker, 1976; Yamamoto et al., 1987; Bourre et al., 1989; Enslen et al., 1991; Frances et al., 1996; Belzung et al., 1998; Carrie et al., 1999 & 2000). Activity of Na, K-ATPase has also been shown to be lower in rats fed diets deficient compared to adequate in C18:3n-3 (Gerbi et al., 1994; Tsutsumi et al., 1995). These studies reinforce the essentiality of C22:6n-3 in rat brain. The importance of C22:6n-3 in brain has given rise to the question of whether C18:3n-3 is sufficient to enable synthesis of adequate amounts of C22:6n-3 for optimal brain growth and development?

Studies with isolated brain cells have provided evidence that astrocytes (glial cells) and cerebroendothelial cells but not neuronal cells can synthesize C20:4n-6 and C22:6n-3 from C18:2n-6 and C18:3n-3, respectively (Moore et al., 1991). Further work has also shown that neuronal cells take up C22:6n-3 released by astrocytes and cerebroendothelial cells and incorporate C22:6n-3 into neural plasma membranes (Moore, 1993; Delton-Vandenbroucke et al, 1997). It is not known whether C20:4n-6 and C22:6n-3 are synthesized from precursors by astrocytes and cerebroendothelial cells in amounts that can meet neuronal cell requirements for DHA *in vivo*.

In weanling rats, increasing maternal dietary C18:3n-3 by decreasing the C18:2n-6 to C18:3n-3 fatty acid ratio from 7.3:1 to 4:1 increased the C22:6n-3 content only in neuronal cell PE from the cerebellum during the first two weeks of life. Other phospholipids from frontal or hippocampal regions were not significantly affected (Jumpsen et al., 1997a & 1997b). Recently, it has been shown that increasing maternal dietary C18:3n-3 by lowering the C18:2n-6 to C18:3n-3 fatty acid ratio does not significantly increase the C22:6n-3 content in PC, PE, and PS of neuronal cells from whole brain of two-week-old rat pups (**Chapter III**). Taken together, these studies suggest that astrocytes and cerebroendothelial cells may not synthesize enough C22:6n-3 from C18:3n-3 to provide for maximal levels of C22:6n-3 in plasma membrane phospholipids of neuronal cells in brain.

It is known that diets supplemented with C22:6n-3 fed to dams or directly to suckling animals enrich the C22:6n-3 content of brain phospholipids (Galli et al., 1971; Anderson et al., 1990). It is not known if maternal dietary C18:3n-3 compared to C22:6n-3 can significantly increase the C22:6n-3 content of both neuronal and glial cell phospholipids from whole brain of rats. Therefore, the present study used neonatal rat brains at two weeks of age, before consumption of solid food, to test the hypothesis that dietary C22:6n-3 but not C18:3n-3 will significantly increase the C22:6n-3 content of both neuronal and glial cell phospholipids of whole brain. The novelty of this study is that it examines: (1) high levels of maternal dietary C18:3n-3 intake and (2) the C22:6n-3 content of both neuronal and glial cells isolated from whole brain.

B. MATERIALS AND METHODS

1. Animals and Diets

Breeding of Sprague-Dawley rats have been described in **Chapter III**. Pups received only maternal milk. Pups were sacrificed at two weeks of age. One entire litter of rat pups fed the same diet was sexed and weighed prior to decapitation. Excised brains were placed in ice-cold 0.32 mol/L sucrose. Six brains from the same sex were pooled per sample. Stomach contents of three rats from each litter were also removed and analyzed for fatty acid composition to represent the composition of maternal milk. Three litters per diet treatment were used.

The basal diet fed to the dams contained 20% (wt/wt) fat (Table 5.1). Diet fats were formulated to approximate the fatty acid composition of an existing infant formula providing an C18:2n-6 to C18:3n-3 fatty acid ratio of 7.3 to 1 (medium LNA) (Jumpsen et al., 1997a and 1997b). This fat blend served as the control fat treatment. An C18:2n-6 to C18:3n-3 fatty acid ratio of 21.6:1 (low LNA) was obtained by addition of corn oil to the medium LNA diet. The diet with a C18:2n-6 to C18:3n-3 fatty acid ratio of 1 to 1 (high LNA) was obtained by the addition of flaxseed oil to the medium LNA diet. The C22:6n-3 (DHA) diet was achieved by the addition of 0.6% (wt/wt) C22:6n-3 to the mediun LNA diet. The C20:4n-6 (AA) + 22:6n-3 diet was achieved by the addition of 1% (wt/wt) C20:4n-6 and 0.6% (wt/wt) C22:6n-3 to the medium LNA diet. The AA and DHA triglycerides utilized were obtained from single cell oils (Martek Biosciences, Columbia, MD). The amount C20:4n-6 (1%) and C22:6n-3 (0.6%) used in this study has been shown by Clandinin et al. (1989) to reflect the C20:4n-6 and C22:6n-3 content found in human milk. The five diets were nutritionally adequate, providing for all known essential nutrient requirements as described earlier (Clandinin & Yamashiro, 1980).

To minimize any changes in sample composition due to fatty acid oxidation, the diets were sealed under nitrogen and stored in a freezer at -30°C in darkness. Every day the required amount of diet was taken out, mixed, and placed in individual feed cups.

Table 5.1 Fatty Acid Composition of Experimental Diets Fed to Lactating Dams at Two-Weeks of Age

DIET FAT:	Low LNA[1]	Medium LNA[2]	High LNA[3]	DHA[4]	AA+DHA[5]
		Fatty Acid Composition (% wt/wt)			
C12:0	6.4	8.4	6.0	7.5	7.7
C14:0	3.8	5.2	3.6	5.3	5.3
C16:0	12.2	14.0	11.4	14.6	14.5
C18:0	9.8	7.4	6.4	7.3	7.4
C18:1n-7+n-9	29.2	39.9	33.8	38.6	39.4
C18:2n-6	34.5	17.2	17.6	16.2	16.5
C18:3n-3	1.6	2.2	17.5	1.8	1.9
C20:4n-6	nd	nd	nd	nd	1.0
C20:5n-3	nd	nd	nd	nd	nd
C22:6n-3	nd	nd	nd	0.6	0.6

[1] Low LNA diet was obtained by the addition of corn oil to the medium LNA diet.
[2] Medium LNA diet approximates the fatty acid composition used in SMA® infant formula.
[3] High LNA diet was obtained by the addition of flaxseed oil to the medium LNA diet.
[4] DHA was obtained by the addition of 0.6% C22:6n-3 triglyceride to the medium LNA diet.
[5] AA + DHA was obtained by addition of 1% C20:4n-6 and 0.6% C22:6n-3 triglyceride to the medium LNA diet.
Nd = not detected.

2. Isolation of Neuronal and Glial Cells from Whole Brain

Neuronal and glial cells were isolated from whole brain (cerebrum and cerebellum) according to the method described by Sellinger & Azcurra (1974). Briefly, pooled brains were placed in beakers containing 7.5% (wt/vol.) polyvinylpyrrolidone and

10 mmol $CaCl_2$ /L at pH 4.7 and 25°C. Brain tissue was minced and poured into a 20 mL plastic syringe, fitted with a reusable filter unit (Millipore, Swinnex disc holder, 25mm). The sample was pressed, three times each, through a series of combined nylon mesh filters. The final filtrate volume was adjusted, then layered on a two-step sucrose gradient of 1.0 mol/L and 1.75 mol/L. Gradients were centrifuged in a Beckman SW-28 rotor at 41,000 g for 30 min at 4°C.

Neuronal cell bodies were recovered in the pellet. Glial cells were obtained at the interface of 1.0 mol/L and 1.75 mol/L sucrose. Aliquots of each cell type were stained with methylene blue and examined for purity under a light microscope (Zeiss, 1600X; Appendix 1). Gel electrophoresis and immunoblotting were performed to ensure purity of cell fractions prepared by these procedures (Jumpsen et al., 1997a and 1997b). Proteins isolated from neuronal and glial cells were compared by gel electrophoresis and immunoblotting to neurofilament and glial fibrillary acid protein standards. Neuronal and glial cells isolated should only contain neurofilament proteins and glial fibrillary acid proteins, respectively.

3. Lipid Extraction and Fatty Acid Analysis

The neuronal and glial cell lipid was extracted by a modified Folch method (Folch et al., 1957). Individual phospholipids from neuronal and glial cells were separated by thin-layer chromatography (Touchstone et al., 1980) and fatty acid methyl esters were prepared following the method of Morrison and Smith (1964). Fatty acid methyl esters were analyzed by automated gas-liquid chromatography as described in **Chapter III**.

4. Statistical Analysis

The effect of diet treatment and cell type on the C20:4n-6 and C22:6n-3 content of neuronal and glial cell PC, PE, PI, and PS was assessed by a two-way analysis of variance (ANOVA) procedure using the SAS™ package, version 6.11 (SAS™ Institute Inc., 1988). Significant differences between diet treatments and cell type were determined by a Duncan's multiple range test at a significance level of $p<0.05$ after a significant ANOVA (Steel & Torrie, 1960). Values are expressed as mean ± SEM for n=6. Two-way

ANOVA procedures were performed on six diet treatments (including a C18:2n-6 to C18:3n-3 fatty acid ratio of 4.4:1), however, in this chapter statistical analysis on five diet treatments were presented for the fatty acid composition of neuronal cell phospholipid fractions in the figures and tables.

C. RESULTS

1. Whole Body and Brain Weights

Whole body and brain weights were not significantly different for male and female rat pups (data not shown), hence statistical analyses to test subsequent effects of diet treatments were combined for both sexes. The whole body and brain weights were not significantly different among the three experimental diet treatments, indicating that whole body and tissue growth in the two-week-old rat pups is not different between diets with or without C22:6n-3. Final body weights were (mean ± SEM): 35.8 ± 1.0 g, 35.9 ± 1.0 g, 35.6 ± 1.3 g, 36.1 ± 0.5 g, and 36.1 ± 0.5 g for low LNA, medium LNA, high LNA, DHA, and AA + DHA diet treatments, respectively. Final brain weights were (mean ± SEM): 1.2 ± 0.1 g, 1.2 ± 0.1 g, 1.1 ± 0.1 g, 1.2 ± 0.1g, 1.2 ± 0.1 g for low LNA, medium LNA, high LNA, DHA, and AA + DHA diet treatments, respectively. Differences in fertility were not observed between the dietary treatments.

2. Purity of Neuronal and Glial Cell Preparations from Whole Brain

Neuronal and glial cell preparations contained only minor cross contamination (≈5%) from cell membrane fragments and microvessels as determined by microscopic examination. The presence of neurofilament in neuronal samples and glial fibrillary acid protein in glial cells samples was previously verified by gel electrophoresis and immunoblotting (Jumpsen et al., 1997a and 1997b). These results indicate that the cell preparations are primarily neuronal and glial cells.

3. Fatty Acid Composition of Stomach Contents

The rat pup stomach contents at day 14 of life contained no particulates indicative of diet consumption and therefore reflected the composition of their dams' milk. The fatty acid composition of stomach contents of rat pups was analyzed. These analyses have been shown to be similar to dams' milk composition (Nouvelot et al., 1983; Yonekubo et al., 1993; Lien et al., 1994; Jumpsen et al., 1997a and 1997b). The dietary C18:3n-3 or C22:6n-3 fed to the dams altered the stomach contents of the rat pups (Table 5.2) indicating that the dietary fat fed in the present experiment produced similar changes in the fat composition of the dams' milk.

Table 5.2 The Content of Fatty Acids in the Stomach of Rat Pups at Two Weeks of Age[1]

DIET FAT:	Low LNA[1]	Medium LNA[2]	High LNA[3]	DHA[4]	AA+DHA[5]
		Fatty Acid Composition	(% wt/wt)		
C10:0	7.9 ± 2.1^{a}	7.2 ± 0.8^{a}	6.9 ± 2.2^{a}	6.7 ± 0.7^{a}	6.2 ± 0.8^{a}
C12:0	10.9 ± 1.1^{c}	15.1 ± 0.4^{a}	10.8 ± 0.6^{c}	13.8 ± 0.2^{b}	12.2 ± 0.1^{b}
C14:0	7.3 ± 0.3^{c}	15.3 ± 0.5^{a}	7.2 ± 0.3^{c}	9.3 ± 0.1^{b}	9.2 ± 0.1^{b}
C16:0	12.6 ± 0.9^{c}	17.7 ± 0.7^{a}	12.9 ± 0.4^{c}	14.9 ± 0.2^{b}	15.9 ± 0.2^{b}
C18:0	4.4 ± 0.4^{a}	3.4 ± 0.1^{b}	4.8 ± 0.2^{a}	4.6 ± 0.1^{a}	4.8 ± 0.1^{b}
C18:1n-7+n-9	28.2 ± 1.5^{a}	24.7 ± 0.7^{b}	26.7 ± 1.0^{b}	31.5 ± 0.8^{a}	29.2 ± 0.6^{a}
C18:2n-6	24.7 ± 0.1^{a}	12.5 ± 0.1^{b}	13.7 ± 0.1^{a}	13.8 ± 0.2^{a}	13.1 ± 0.1^{b}
C18:3n-3	1.1 ± 0.0^{c}	1.6 ± 0.0^{b}	8.8 ± 0.1^{a}	1.5 ± 0.0^{b}	1.6 ± 0.0^{b}
C20:4n- 6	1.0 ± 0.1^{a}	0.5 ± 0.0^{b}	0.5 ± 0.1^{b}	0.5 ± 0.0^{b}	1.1 ± 0.1^{a}
C22:6n-3	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}	0.2 ± 0.0^{b}	0.6 ± 0.0^{a}	0.7 ± 0.0^{a}
C18:2n-6:C18:3n-3	22.5	7.8	1.6	9.2	8.2

[1] Values are mean ± SEM with n=9 for each experimental diet. For each horizontal set of values, those that have the same superscript letters (a, b, or c) are not significantly different. Those that have different letters are significantly different, at $p<0.05$.

[2] Low LNA diet was obtained by the addition of corn oil to the medium LNA diet.

[3] Medium LNA diet approximates the fatty acid composition used in SMA® infant formula.

[4] High LNA diet was obtained by the addition of flaxseed oil to the medium LNA diet.

[5] DHA was obtained by the addition of 0.6% C22:6n-3 triglyceride to the medium LNA diet.

[6] AA + DHA was obtained by addition of 1% C20:4n-6 and 0.6% C22:6n-3 triglyceride to the medium LNA diet.

4. Neuronal and Glial Cells Phospholipid Fatty Acid Composition

a) Phosphatidylethanolamine

The analysis of the fatty acid profile in neuronal and glial cell PE from whole brain demonstrated that C16:0, C18:0, C20:4n-6, and C22:6n-3 (11-16%, 24-26%, 16-20%, and 16-29% of the total fatty acids, respectively) were the predominant fatty acids. There was no significant difference in the C20:4n-6 content of PE between neuronal and glial cells (Figure 5.1). However, the C22:6n-3 content of PE was significantly different between neuronal and glial cells ($p<0.05$) (Figure 5.1). Glial cells contained a significantly greater amount of C22:6n-3 in PE than neuronal cells. The C20:4n-6 content of PE from both cell types was not significantly different between the diet treatments (Figure 5.1). The C22:6n-3 content of PE in neuronal cells was significantly increased by feeding the DHA and AA + DHA diet treatments compared to feeding diets providing low LNA, medium LNA, high LNA (Figure 5.1). The C22:6n-3 content of glial cell PE was significantly increased with animals fed the DHA diet (Figure 5.1). The C22:6n-3 content of neuronal cell PE was (mean ± SEM; %wt/wt); 21.2 ± 0.6%, 17.4 ± 2.2 %, 15.7 ± 1.1%, 26.2 ± 1.6%, and 26.8 ± 0.6%, for animals fed the low LNA, medium LNA, high LNA, DHA, and AA + DHA diet treatments, respectively (Figure 5.1). The C22:6n-3 content of glial cell PE was (mean ± SEM; %wt/wt); 17.7 ± 1.3%, 20.9 ± 2.4%, 25.1 ± 0.3%, 31.6 ± 0.2%, and 23.8 ± 0.5%, for animals fed the low LNA, medium LNA, high LNA, DHA, and AA + DHA diet treatments, respectively (Figure 5.1).

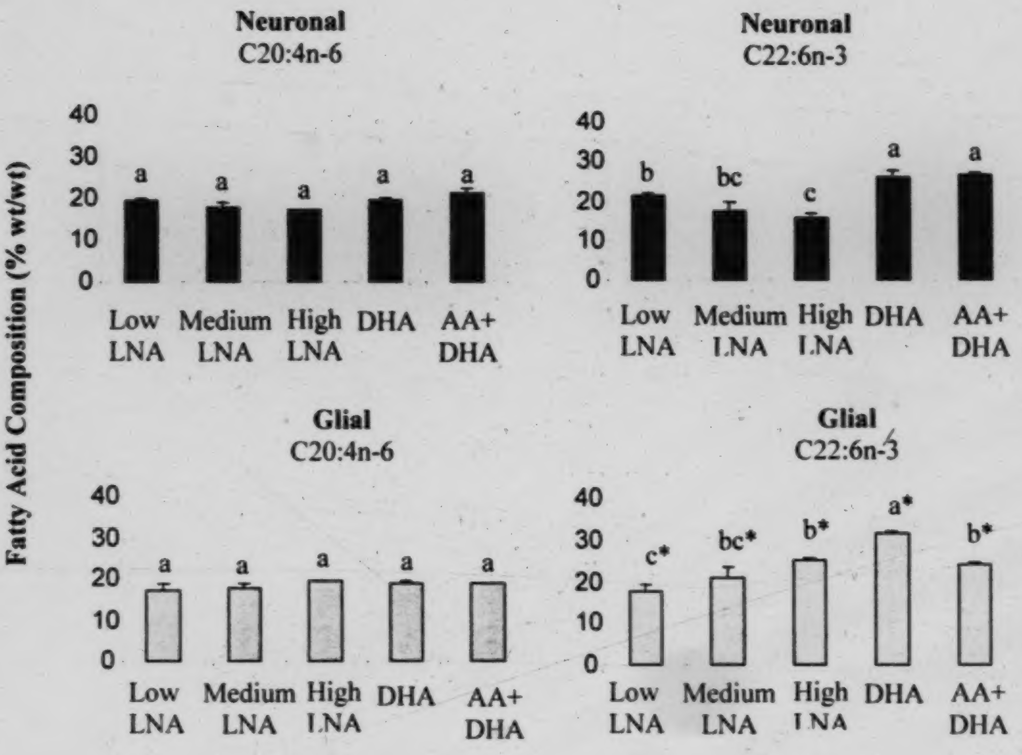

Figure 5.1 Fatty Acid Composition of Neuronal and Glial Cell Phosphatidylethanolamine * Cell Type Difference at $p<0.05$

b) Phosphatidylserine

The major fatty acids in neuronal and glial cells PS from whole brain were C16:0, C18:0, and C22:6n-3 (7-20%, 36-44%, and 14-33% of the total fatty acids, respectively). The C20:4n-6 content of PS between neuronal and glial cells was not significantly different. The C22:6n-3 content of PS was different between neuronal and glial cells with glial cells containing a significantly greater amount of C22:6n-3 than neuronal cells ($p<0.05$; Figure 5.2). The C20:4n-6 content of PS from both cell types was not significantly different between the diet treatments (Figure 5.2). Feeding the DHA and AA + DHA diet significantly increased the C22:6n-3 content of neuronal PS compared to the diets providing a C18:2n-6 to C18:3n-3 fatty acid ratio of 21.6:1 and 1:1 but not 7.8:1

(p<0.0001; Figure 5.2). The C22:6n-3 content of neuronal cell PS was (mean ± SEM; %wt/wt); 20.0 ± 0.7%, 28.0 ± 0.7% , 14.1 ± 2.9% , 29.5 ± 1.4%, and 29.5 ± 0.9%, for animals fed low LNA, medium LNA, and high LNA, DHA, and AA + DHA diet treatments, respectively (Figure 5.2). In glial cells, the C22:6n-3 content of PS was significantly increased with animals fed DHA and AA + DHA diet compared to the animals fed low and high LNA, but not the mediun LNA diets (p<0.0001; Figure 5.2). The C22:6n-3 content of glial cell PS was (mean ± SEM; %wt/wt): 23.2 ± 0.6%, 25.5 ± 3.2%, 22.7 ± 1.5%, 34.1 ± 1.6%, and 31.3 ± 0.5%, for animals fed low LNA, medium LNA, high LNA, DHA, and AA + DHA diet treatments, respectively (Figure 5.2).

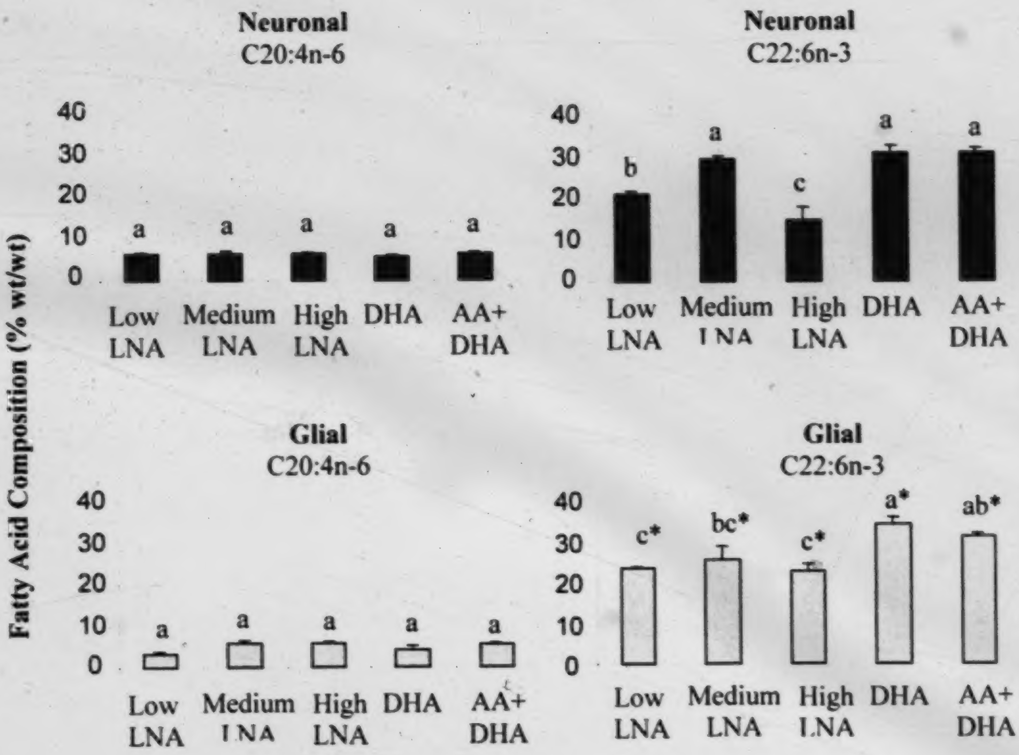

Figure 5.2 Fatty Acid Composition of Neuronal and Glial Cell Phosphatidylserine

* Cell Type Difference at p<0.05

c) Phosphatidylcholine and Phosphatidylinositol

The predominant fatty acids observed in PC were C16:0, C18:0, and C18:1 (47-52%, 9-16%, 18-22% of the total fatty acids, respectively; Table 5.3). PC C20:4n-6 and C22:6n-3 content was significantly different between neuronal and glial cells. Neuronal cells had greater amounts of C20:4n-6 and C22:6n-3 in PC than glial cells (Table 5.3). Feeding a maternal diet providing a fatty acid ratio of C18:2n-6 to C18:3n-3 from 21.6:1 to 1:1, and DHA and AA + DHA did not significantly increase the C20:4n-6 and C22:6n-3 content of PC in neuronal and glial cells (Table 5.3). In neuronal and glial cell PI, the major fatty acids were C16:0, C18:0, C18:1, and C20:4n-6 (8-15%, 28-37%, 5-12%, and 19-35% of total fatty acids, respectively; Table 5.4). C20:4n-6 but not the C22:6n-3 content was significantly different between neuronal and glial cells with neuronal cells containing significantly more C20:4n-6 in PI than glial cells (Table 5.4).

Table 5.3 Effect of Low to High Dietary C18:3n-3 Compared to Feeding C22:6n-3 on the Fatty Acid Composition of Neuronal and Glial Cell Phosphatidylcholine[1]

DIET FAT:	Low LNA[2]		Medium LNA[3]		High LNA[4]		DHA[5]		AA + DHA[6]	
	Fatty Acid (% wt/wt)									
	Neuronal	Glial	Neuronal	Glial	Neuronal	Glial	Neuronal	Glial	Neuronal	Glial
C16:0	50.4±0.6^{a}	46.8±1.6^{a}	46.7±0.9^{a}	51.5±0.4^{a}	50.6±0.0^{a}	48.6±0.4^{a}	50.7±0.3^{a}	50.8±0.5^{a}	48.3±1.2^{a}	52.0±0.9^{a}
C18:0	13.3±0.3^{a}	12.8±1.2^{a}	15.5±0.4^{a}	8.9±0.0^{a}	13.4±0.2^{a}	14.4±0.9^{a}	9.4±0.5^{b}	10.5±1.0^{b}	10.7±1.4^{b}	10.5±0.3^{b}
C18:1n-9	15.3±0.1^{a*}	17.1±0.9^{a}	15.1±0.3^{a*}	16.4±0.0^{a}	15.2±0.0^{a*}	16.6±0.3^{a}	15.9±1.3^{a*}	17.1±0.2^{a}	14.1±3.6^{a*}	16.5±0.3^{a}
C18:2n-6	1.2±0.0^{a}	2.5±0.8^{a}	1.2±0.0^{b}	1.2±0.0^{b}	1.2±0.0^{ab}	1.4±0.1^{ab}	1.5±0.2^{ab}	1.3±0.1^{ab}	1.2±0.1^{b}	0.9±0.1^{a}
C18:3n-3	0.0±0.0^{a}	0.1±0.1^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
C20:5n-3	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.1±0.1^{a}
C22:5n-3	0.1±0.0^{a*}	0.2±0.2^{a}	0.1±0.0^{a*}	0.1±0.0^{a}	0.2±0.0^{a*}	0.1±0.1^{a}	0.1±0.1^{a*}	0.1±0.0^{a}	0.1±0.0^{a*}	0.1±0.0^{a}
C22:6n-3	3.2±0.2^{a*}	3.1±0.5^{a}	3.6±0.2^{a*}	3.3±0.1^{a}	3.1±0.4^{a*}	2.6±0.1^{a}	3.9±0.6^{a*}	3.4±0.4^{a}	4.3±0.5^{a*}	2.9±0.3^{a}
C20:4n-6	7.0±0.1^{a*}	6.6±0.6^{a}	7.2±0.1^{a*}	7.0±0.1^{a}	6.5±0.1^{a*}	6.8±0.3^{a}	7.5±0.6^{a*}	6.5±0.7^{a}	8.5±0.8^{a*}	6.5±0.6^{a}
C22:4n-6	0.9±0.1^{a*}	0.4±0.3^{a}	1.2±0.1^{a*}	0.6±0.1^{a}	0.8±0.0^{a*}	0.6±0.1^{a}	0.8±0.1^{a*}	0.7±0.1^{a}	1.0±0.1^{a*}	0.7±0.1^{a}
C22:5n-6	0.4±0.0^{a}	0.3±0.1^{a}	0.3±0.0^{a}	0.1±0.0^{a}	0.2±0.0^{a}	0.1±0.0^{a}	0.2±0.0^{a}	0.1±0.0^{a}	0.3±0.1^{a}	0.1±0.0^{a}

[1] Values are mean ± SEM with n=6 for each experimental diet. For each horizontal set of values within a cell type, those that have the same superscript letters (a or b) are not significantly different. Those that have different letters are statistically significant different at $p<0.05$.

[2] Low LNA diet was obtained by mixing safflower oil into the medium LNA diet.

[3] Medium LNA diet approximates the fatty acid composition used in SMA® infant formula.

[4] High LNA diet was obtained by the addition of flaxseed oil to the medium LNA diet.

[5] DHA was obtained by the addition of 0.6% C22:6n-3 triglyceride to the medium LNA diet.

[6] AA + DHA was obtained by addition of 1% 20:4n-6 and 0.6% C22:6n-3 triglyceride to the medium LNA diet.* Cell type difference at $p<0.05$.

Table 5.4 Effect of Low to High Dietary C18:3n-3 Compared to Feeding C22:6n-3 on the Fatty Acid Composition of Neuronal and Glial Cell Phosphatidylinositol[1]

DIET FAT:	Low LNA[2]		Medium LNA[3]		High LNA[4]		DHA[5]		AA + DHA[6]	
	Fatty Acid (%wt/wt)									
	Neuronal	Glial	Neuronal	Glial	Neuronal	Glial	Neuronal	Glial	Neuronal	Glial
C16:0	13.8 ± 1.3^{ab}	13.3 ± 1.3^{ab}	7.4 ± 1.7^{bc}	14.3 ± 2.7^{bc}	15.2 ± 0.3^{a}	15.1 ± 1.0^{a}	10.2 ± 1.6^{c}	8.7 ± 0.6^{c}	11.7 ± 0.7^{bc}	11.5 ± 0.8^{bc}
C18:0	28.1 ± 1.3^{b}	28.1 ± 1.1^{b}	34.7 ± 0.5^{a}	34.8 ± 1.4^{a}	31.0 ± 1.0^{b}	28.4 ± 0.7^{b}	33.6 ± 0.0^{a}	33.8 ± 0.8^{a}	36.7 ± 2.9^{a}	32.4 ± 2.1^{a}
C18:1n-9	8.1 ± 0.6^{b}	9.7 ± 1.9^{b}	6.1 ± 0.7^{b}	6.0 ± 0.5^{b}	12.3 ± 1.0^{a}	11.3 ± 1.5^{a}	6.7 ± 0.8^{b}	5.2 ± 0.4^{b}	5.1 ± 1.5^{b}	6.8 ± 0.8^{b}
C18:2n-6	0.8 ± 0.0^{a}	1.8 ± 0.3^{a}	0.7 ± 0.1^{b}	0.4 ± 0.1^{b}	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	0.5 ± 0.0^{b}	0.5 ± 0.1^{b}	0.4 ± 0.1^{b}	0.4 ± 0.1^{b}
C18:3n-3	0.3 ± 0.3^{a}	0.3 ± 0.1^{a}	0.1 ± 0.0^{b}	0.0 ± 0.0^{b}	0.1 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.2 ± 0.2^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
C20:5n-3	$0.4\pm0.3^{a*}$	1.7 ± 0.4^{a}	$0.1\pm0.0^{b*}$	0.1 ± 0.0^{b}	$0.1\pm0.0^{b*}$	0.2 ± 0.1^{b}	$0.2\pm0.0^{a*}$	1.1 ± 0.8^{a}	$0.2\pm0.0^{a*}$	0.9 ± 0.7^{a}
C22:5n-3	$0.2\pm0.0^{a*}$	0.6 ± 0.3^{a}	$0.2\pm0.0^{a*}$	0.4 ± 0.3^{a}	$0.4\pm0.0^{a*}$	0.5 ± 0.1^{a}	$0.2\pm0.0^{a*}$	0.1 ± 0.0^{a}	$0.2\pm0.0^{a*}$	0.7 ± 0.4^{a}
C22:6n-3	11.1 ± 1.6^{a}	8.7 ± 1.9^{a}	6.2 ± 0.3^{b}	7.2 ± 0.9^{b}	10.7 ± 0.5^{a}	13.1 ± 1.5^{a}	6.8 ± 0.7^{a}	13.7 ± 0.6^{a}	9.8 ± 0.7^{ab}	7.9 ± 1.1^{ab}
C20:4n-6	$26.5\pm1.6^{a*}$	21.8 ± 1.7^{a}	$35.4\pm2.3^{a*}$	27.8 ± 4.0^{a}	$19.0\pm1.4^{a*}$	20.1 ± 1.5^{a}	$35.1\pm1.6^{a*}$	27.7 ± 1.7^{a}	$28.5\pm1.3^{a*}$	30.5 ± 1.8^{a}
C22:4n-6	3.1 ± 0.5^{a}	2.1 ± 0.5^{a}	1.8 ± 0.1^{a}	1.3 ± 0.4^{a}	3.0 ± 0.1^{a}	3.5 ± 0.4^{a}	1.9 ± 0.3^{a}	1.8 ± 0.1^{a}	2.6 ± 0.1^{a}	2.1 ± 0.3^{a}
C22:5n-6	1.3 ± 0.2^{a}	0.8 ± 0.3^{a}	0.5 ± 0.1^{a}	0.8 ± 0.5^{a}	0.8 ± 0.0^{a}	1.0 ± 0.1^{a}	0.3 ± 0.1^{a}	0.4 ± 0.1^{a}	0.7 ± 0.0^{a}	0.6 ± 0.1^{a}

[1] Values are mean ± SEM with n=6 for each experimental diet. For each horizontal set of values within a cell type, those that have the same superscript letters (a or b) are not significantly different. Those that have different letters are statistically significant different at $p<0.05$.

[2] Low LNA diet was obtained by mixing safflower oil into the medium LNA diet.

[3] Medium LNA diet approximates the fatty acid composition used in SMA® infant formula.

[4] High LNA diet was obtained by the addition of flaxseed oil to the medium LNA diet.

[5] DHA was obtained by the addition of 0.6% C22:6n-3 triglyceride to the medium LNA diet.

[6] AA + DHA was obtained by addition of 1% 20:4n-6 and 0.6% C22:6n-3 triglyceride to the medium LNA diet.* Cell type difference at $p<0.05$.

D. DISCUSSION

The present results establish the hypothesis that dietary C22:6n-3 but not increased intake of C18:3n-3 will significantly increase the C22:6n-3 content of both neuronal and glial cell phospholipids from whole brain. These observations extend previous information (Jumpsen et al., 1997) by establishing that maternal dietary C22:6n-3 is more effective at increasing the C22:6n-3 content of whole brain neuronal and glial cell membrane phospholipid in two-week-old rat pups than feeding low or high levels of maternal C18:3n-3.

The results from this study are in accordance with other studies showing that rat brain uses C22:6n-3 instead of C18:3n-3 to maintain the C22:6n-3 content of membrane in phospholipids (Sinclair, 1975; Anderson & Connor, 1988; Edmond et al., 1998). This study has shown that C22:6n-3 can be taken up by the brain and used for neuronal and glial cell membrane phospholipids synthesis.

Feeding low or high maternal dietary C18:3n-3 did not significantly increase the C22:6n-3 content in PE, PS, and PC from neuronal and glial cells compared to feeding diets containing C22:6n-3. The lack of significant increase in C22:6n-3 with rat pups fed the high LNA diet may be explained by delta-6 desaturase, the rate-limiting enzyme in the synthesis of C22:6n-3, being not fully active at two weeks of age (Bernhart & Sprecher, 1975). Rodent studies have demonstrated that liver (Nouvelot et al, 1986), astrocytes (Moore et al., 1990 and 1991), choroid plexus (Bourre et al, 1997) and microvessel endothelial cells (Delton-Vandenbroucke et al., 1999) have delta-6 desaturase activity and that C18:3n-3 is converted to C22:6n-3 in these cell types (Ravel et al. 1985; Sanders and Rana, 1987; Bourre et al. 1990). Whether rat pups at two weeks of age are able to convert sufficient amounts of C18:3n-3 into C22:6n-3 to provide for maximal incorporation of C22:6n-3 into neuronal cell membrane phospholipid synthesis is not known. The findings from the present study are in agreement with our previous study showing that low to high maternal C18:3n-3 diets does not significantly increase the C22:6n-3 content of neuronal cell membrane phospholipids (**Chapter III**). In two-week-old rat pups, the metabolic fate of feeding a high maternal C18:3n-3 diet is deposition of C18:3n-3 in the skin, including subcutaneous fat and adipose tissue, of two-week-old rat pups (**Chapter IV**).

The C20:4n-6 content of neuronal and glial cells PE, PS, PC, and PI was not significantly different among rat pups fed the different diet treatments at two-weeks of age. The reason for the non-significant difference in C20:4n-6 content of individual phospholipids of rats fed the different diet treatments may be explained by the C20:4n-6 present in the stomach contents (Table 2). It is possible that the level of the C20:4n-6 (>0.5%) present in the stomach contents of rat pups may be sufficient to prevent a significant decrease in C20:4n-6 content of PE, PS, PC, and PI from neuronal and glial cells of two-week old rat pups.

In early postnatal rat brain, PE and PS constitute approximately 30% and 6% of the total phospholipids, respectively (Green and Yavin, 1996). PE and PS in brain are particularly enriched in C22:6n-3 (Breckenridge et al., 1972; Salem et al., 1980; Martinez, 1989) and contain most of the C22:6n-3 (~92%) esterified into the total brain phospholipids by the first week of postnatal life (Green and Yavin, 1996). Therefore, any changes in whole brain neuronal and glial cell C22:6n-3 content caused by dietary fat treatments used in this study should be detected in PE and PS.

Glial cells contained significantly more C22:6n-3 in PE and PS than neuronal cells. Neuronal cells contained greater amounts of C22:6n-3 in PC and C20:4n-6 in PC and PI than glial cells. This difference between neuronal and glial cells C22:6n-3 and C20:4n-6 content is similar to previous findings (Jumpsen et al. 1997a & 1997b) in which differences were observed between C22:6n-3 and C20:4n-6 content in phospholipids of both cell types in the frontal, cerebellum, and hippocampal regions of brain of developing rat pups. Thus, it may be concluded that neuronal and glial cells respond differently to dietary fat treatment.

The functional implications of an increase in C22:6n-3 content of PE and PS of neuronal and glial cells with rat pups fed diets with C22:6n-3 at two-week-old rat pups is not known. Investigations of functional changes associated with an increase in C22:6n-3 content of PE and PS from neuronal and glial cells would be of great interest since modification of the PUFA content of cell membranes has a large impact on membrane properties and the functioning of a variety of membrane-associated proteins such as transporters, enzymes, and receptors (reviewed by Spector & Yorek, 1985 and Clandinin, 1997). PS is involved in variety of cell functions (Salem & Niebylski, 1995) such as signal transduction via its activation of several protein kinase C isoforms (Bell & Burns, 1991) or Raf-1 kinase to cell membranes (Ghosh et al., 1996), modulation of synaptosomal benzodiazepine receptors (Levi deStein et al., 1989), and increases synaptic efficiency (Borghese et al., 1993). Therefore, it is likely that the diet-induced alterations in neuronal and glial cell C22:6n-3 content observed in PS in the present study may have a physiological impact.

In conclusion, the findings from this study demonstrate that maternal C22:6n-3 is more effective in increasing the C22:6n-3 content of brain PE and PS in neuronal and glial cells in two-week-old rat pups than maternal dietary levels of C18:3n-3. The results of this study in rats suggest that neonates will achieve increased brain C22:6n-3 levels more effectively if preformed C22:6n-3 is fed instead of increased intake of C18:3n-3.

In both two-week-old rat pups and six-month-old human infants, early gliogenesis and macroneurogenesis are completed while microneurogenesis, late gliogenesis, and myelination are continuing in both species during this period (reviewed by Morgane et al., 1993). Rat pups and human infants have a similar metabolic pathway for synthesis of C20:4n-6 and C22:6n-3 via desaturation and elongation of precursors. Rodents have a markedly higher desaturase activity compared to human infants (Cunnane et al., 1984; Horrobin et al., 1984). Thus, it is reasonable to speculate that infants may produce relatively less C22:6n-3 in neuronal and glial cell phospholipids when compared to C22:6n-3 production by rat pups fed the same level of dietary intake of C18:3n-3.

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CHAPTER VI. DIETARY LINOLENIC AND DOCOSAHEXAENOIC ACID ALTER SYNAPTIC PLASMA MEMBRANE PHOSPHOLIPID FATTY ACID COMPOSITION AND SODIUM-POTASSIUM ADENOSINE TRIPHOSPHATASE KINETICS IN DEVELOPING RATS

A. INTRODUCTION

The SPM is comprised of phospholipids with high levels of PUFAs, particularly, C22:6n-3 (Cotman et al., 1969; Breckenridge et al., 1972; Sun & Sun, 1972; Foot et al., 1982; Hargreaves et al., 1989). Histological (Mashanskii et al., 1969) as well as biochemical studies have shown that Na, K-ATPase (EC 3.6.1.37) is associated with brain SPM (Kurokawa et al., 1965; Cotman et al., 1969). Na, K-ATPase in SPM has a specific and distinct function in maintenance of cation gradients across neuronal membrane, impulse propagation, release and uptake of neurotransmitters (Skou, 1957; Schwartz et al., 1975, Gloor, 1997; da Silva et al., 1999). Past research has shown that activity of SPM Na, K-ATPase can be modulated by phospholipids and cholesterol (Goldman & Albers, 1973) as well as by the fatty acyl groups of the SPM phospholipids (Sun & Sun, 1974; Kimelberg & Papahadjopoulos, 1974; Srivivasarao et al., 1997; Gerbi & Maxient, 1999). Alterations in diet fat have been shown to induce changes in the phospholipid content (Foot et al., 1982; Hargreaves and Clandinin, 1987) and fatty acyl composition of SPM (Foot et al., 1982), accompanied by alterations in activity of membrane-associated enzymes (Sun & Sun, 1974; Foot et al., 1983; Bourre et al., 1989; Tsutsumi et al., 1995; Gerbi & Maxient, 1999), and regulation of neurotransmitters (Delion et al., 1994; Zimmer et al., 1998). Feeding rats and their offspring diets deficient in C18:3n-3 (LNA) dramatically alters the fatty acid composition of SPM phospholipids by decreasing n-3 PUFA and increasing n-6 PUFA, particularly, C22:4n-6 and C22:5n-6 (Salem et al., 1986; Bourre et al., 1984, 1989, & 1993; Gazzah et al., 1993). Long term n-3 deficiency affects membrane-bound enzymes activities (Salem et al., 1986; Bourre et al., 1989; Tsutsumi et al., 1995) decreasing Na, K-ATPase activity at optimal (Bourre et al., 1984) and suboptimal (Tsutsumi et al., 1995) ATP concentrations.

Increasing maternal dietary C18:3n-3 from 1.6% to 17.5% of total fatty acids, by lowering the C18:2n-6 to C18:3n-3 fatty acid ratio from 22:1 to 1:1, does not significantly increase the C22:6n-3 content in PC, PE, and PS of neuronal cells from whole brain of two-week-old rat pups (**Chapter III**). The metabolic fate of feeding a high C18:3n-3 diet is deposition of C18:3n-3 in tissues, particularly, skin (epidermis, dermis, and subcutaneous tissue) (**Chapter IV**). Human and animal studies have shown that feeding preformed C22:6n-3 from fish or single-cell oils can significantly increase the C22:6n-3 content of brain (Jumpsen et al., 1997), erythrocytes, and plasma phospholipids (Makrides et al, 1994; Clandinin et al, 1997). Since C22:6n-3 is enriched in SPM phospholipids, it would be of interest to determine whether change in SPM phospholipid C22:6n-3 content occurring in rats fed diets containing C18:3n-3 or C22:6n-3 has functional consequences.

Thus, the objective of the present study was to investigate if maternal dietary C20:4n-6 (AA) and C22:6n-3 compared to feeding adequate or low levels of C18:3n-3 (LNA) increases SPM cholesterol and phospholipid content, phospholipid C20:4n-6 and C22:6n-3 content, and Na, K-ATPase kinetics in rat pups at two and five weeks of age.

B. MATERIALS AND METHODS

1. Animal Care

Breeding of Sprague-Dawley rats have been described in **Chapter III**. All litters were culled to twelve rat pups following parturition. Rats sacrificed at two weeks of age received only maternal milk. Rats sacrificed at five weeks of age were weaned at three weeks of age to the same diet received by their respective dams.

One entire litter of rat pups fed the same diet was sexed and weighed before decapitation. Birth and weaning weights were not determined. Excised brains were placed in ice-cold 0.32 M sucrose. Six brains from the same sex were pooled per sample for isolation of SPM. Stomach contents of three rats from each litter were also removed and analyzed for fatty acid composition to reflect the composition of maternal milk and diet. Three litters per diet treatment and age were used.

2. Diets

Three semi-synthetic 20% (wt/wt) fat diets were fed (Clandinin & Yamashiro, 1982). The diets differed by the amount or nature of n-6 and n-3 fatty acids (Table 6.1). The control fat diet was formulated to approximate the fatty acid composition of an existing infant formula providing an C18:2n-6 to C18:3n-3 ratio of 7.1:1 (Table 6.1). The low LNA diet was obtained by addition of safflower oil as the diet fat blend. The AA+DHA diet was obtained by addition of C20:4n-6 (ARASCO™) and C22:6n-3 (DHASCO™) triglycerides from single cell oils (Martek Biosciences, Columbia, MD, USA) to the control diet fat blend. These diets were nutritionally adequate, with exception of the low LNA diet (0.04% of energy as C18:3n-3), providing for all known essential nutrient requirements as described in **Chapter III**. To minimize fatty acid peroxidation, the diets were sealed under nitrogen and stored in a freezer at -30°C in darkness. Each day the required amount of diet was mixed thoroughly and placed in individual feed cups.

3. Isolation of Synaptic Plasma Membrane

Rat brains in 0.32 M sucrose were homogenized in 10 volumes of 0.32 M sucrose with 1 mM EDTA, pepstatin A (20 μg/mL), aprotinin (20 μg/mL), trypsin inhibitor (20 μg/mL), phenylmethylsulfonyl fluoride (5 μg/mL), and leupeptin (20 μg/mL), pH 7.4 homogenizing buffer (Nikolova-Karakashian & Merrill, 2000; Cotman, 1974). The homogenate was centrifuged at 3,000 g for 5 min to remove the nuclear fraction (Cotman, 1974). Supernatant was then centrifuged at 10,500 g for 20 min to obtain the crude mitochondrial pellet (Cotman, 1974). The pellet obtained was resuspended in 3 mL of homogenizing buffer, layered over a preformed discontinuous sucrose gradient of 0.8 M and 1.2 M and centrifuged in a swinging-bucket rotor (Beckman SW-28) at 97,000 g for 2 hr (Appendix 2; Cotman, 1974). The band at the interface of 0.8 M and 1.2 M sucrose was diluted and lysed in ice-cold distilled water for 1 hr at 4°C (Cotman, 1974). The SPM was recovered by centrifugation in a fixed angle rotor (Beckman JA-20 rotor) at 19,000 g for 30 min. This pellet was resuspended in homogenizing buffer and used for subsequent analysis of lipid, protein, and Na, K-ATPase (Esmann, 1988). The SPM purity was tested by measuring the RNA content for microsomes (Fleck & Begg, 1965) and succinic dehydrogenase for mitochondrial contamination (Pennington, 1961).

Table 6.1 Fatty Acid Composition of Experimental Diets[a]

Diet Treatment :	Low LNA[T]	Control[†]	AA + DHA[‡]
Fatty acid (% wt/wt)			
C10:0	nd	1.23	1.92
C12:0	nd	11.7	13.1
C14:0	0.11	5.70	6.39
C16:0	6.66	12.5	12.7
C18:0	2.52	6.13	7.27
C18:1n-9	13.6	40.3	37.2
C18:1n-7	0.40	3.21	2.57
C18:2n-6	75.2	17.9	16.5
C18:3n-3	0.09	2.51	2.54
C20:4n-6	nd	nd	1.11
C22:6n-3	nd	nd	0.64
Σ Sat	9.29	37.3	41.3
Σ Mufa	14.0	43.5	39.8
Σ N-6	75.2	17.9	17.6
Σ N-3	0.09	2.51	3.18
C18:2n-6 to C18:3n-3 ratio	835	7.13	6.50

[a] nd = not detected; Σ sat, sum of saturated fatty acids; Σ mufa, sum of monounsaturated fatty acids; Σ n-6, sum of n-6 fatty acids; and Σ n-3, sum of n-3 fatty acids.

[T] Low LNA diet (0.04% of energy) was obtained by the addition of safflower oil as the fat blend.

[†] The control fat diet of C18:2n-6 to C18:3n-3 fatty acid ratio of 7.1:1 approximates the fatty acid composition used in SMA® infant formula.

[‡] The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

4. Lipid Analysis

Total lipids within the SPM fraction were extracted as described by Folch et al. (1957). The lower chloroform phase of the lipid extract was evaporated to dryness under a stream of nitrogen. The residue was resuspended in 2 mL chloroform : methanol (2:1, by vol.) and aliquots were taken for determination of total and individual phospholipid and cholesterol content and fatty acid analysis. Total phospholipid from aliquots of lower phase chloroform : methanol (2:1, by vol.) were determined by spotting the samples on a prewashed silica gel "G" thin-layer chromatography (TLC) plate (20 x 20 cm, Analtech, Newark, DE) and developing the plate in a solvent system containing petroleum ether : diethyl ether : acetic acid (80:20:1, by vol.) for approximately 1 hr (Suh et al., 1996). The total phospholipid band was visualized with 0.1% (wt/vol.) aniline naphthalene sulfonic acid (ANSA) in water. Separation of individual phospholipids was completed on prewashed silica gel, "H" TLC plates (20 x 20 cm, Analtech, Newark, DE). TLC plates were developed in a solvent system containing chloroform : methanol : triethylamine : 1-propanol : 0.25% (wt/vol.) KCl (30:9:18:25:6, by vol.) for approximately 90 min. (Touchstone et al., 1980). TLC plates were air-dried for 5 min. and visualized with 0.1% ANSA.

Total and individual phospholipid content of SPM was determined in triplicate by inorganic phosphorous assay after digestion with 72% perchloric acid at 180 °C for 1 hr (Chen et al., 1956). Cholesterol was measured according to the procedure of Zlatkis & Zak (1969). Individual SPM phospholipid fatty acid methyl esters were prepared with 14% (wt/wt) boron trifluoride in methanol following the method of Morrison & Smith (1964).

5. Fatty Acid Analysis

Fatty acid methyl esters were analyzed by automated gas-liquid chromatography as described in **Chapter III**.

6. Na, K-ATPase Assay

The Na, K-ATPase activity was measured following the method described by Esmann (1988) and Tsutsumi et al. (1995). An aliquot (100µL) of the SPM fraction was incubated at 37 °C for 15 min. with reaction buffer containing 100 mM NaCl, 20mM KCl, 5 mM $MgCl_2$, various concentrations of ATP from 0.5 to 6 mM, and 30 mM histidine buffer, pH 7.4 in the presence and absence of 1 mM ouabain, a specific inhibitor of Na, K-ATPase (Kawamura et al., 1999) in a total volume of 1 mL. The reaction was terminated by adding 0.5 mL 10% (wt/vol.) trichloroacetic acid. After a 10 min centrifugation, 100μL of supernatant was removed and assayed for inorganic phosphate content (Pi). Inorganic phosphate liberated was determined spectrophotometrically (Chen et al., 1956) using K_2HPO_4 as standard. Na, K-ATPase activity was obtained by the difference between total ATPase and Mg^{2+}-ATPase (ouabain-insensitive) activity (Esmann, 1988).

Na, K-ATPase activity is expressed as μmol Pi / mg / hr. The protein concentration in the SPM was estimated by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard. Six different membrane preparations were assayed in triplicate for each diet treatment and age. Data for kinetic analysis was plotted as an Eadie-Hofstee plot and analyzed by a linear regression program (Caspers et al., 1993). The slope (Km) and y-intercepts (Vmax) were determined.

7. Synaptic Plasma Membrane PAGE and Immunoblotting

For analysis of Na, K-ATPase β1-subunit, six different membrane preparations for each diet treatment and age were pooled. SPM protein (20 μg) samples from rats fed either the low LNA, control, or AA + DHA diets at two and five weeks of age were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970) and then transferred to nitrocellulose paper by electroblotting (Towbin et al., 1979). The quantity of protein in each lane as well as the efficiency of transfer was confirmed by staining with Ponceau S. Western blots were probed with an anti-β1 antibody (dilution 1:1000) (Upstate Biotechnology, Lake Placid, NY, USA). The β1-subunit was detected by chemiluminescence (Pierce, Rockfort, Illinois, USA) and visualized on x-ray film (X-OMAT AR™). The protein bands corresponding to the β1-subunit of Na, K-ATPase were quantitated by transmittance densitometry using a Bio-Rad imaging densitometer (Life Science Group, Cleveland, Ohio, USA).

8. Statistical Analysis

The effect of diet treatment and age on body weight, brain weight, SPM lipid content, SPM fatty acid composition, and Na, K-ATPase kinetics was assessed by two-way analysis of variance (ANOVA) procedures using an SAS™ package, version 6.11 (SAS™ Institute, 1988). Significant differences between diet treatments and age were determined by Duncan's multiple range test at a significance level of $p<0.05$ after a significant ANOVA (Steel & Torrie, 1960). Values are expressed as mean ± SEM for n=6.

C. RESULTS

1. Growth Characteristics

Body and brain weights were significantly different for male and female rat pups at two and five weeks of age. Male rats had significantly greater body and brain weights compared to female rats at two and five weeks of age. Body weights for male and female rats at two and five weeks of age were (mean ± SEM): 34.4 ± 0.51 g and 33.0 ± 0.40 g ($p<0.002$) and 140 ± 2.0 g and 134 ± 1.5 g ($p<0.003$), respectively. Brain weights for male and female rats at two and five weeks of age were (mean ± SEM): 1.24 ± 0.00 g and 1.21 ± 0.01 g ($p<0.02$) and 1.73 ± 0.01 g and 1.69 ± 0.01 g ($p=0.05$), respectively. Body and brain weights and brain to body weight ratio, however, were not significantly different among diet groups at two and five weeks of age (data not shown), indicating that body and brain growth in rats are not affected by adequate or low LNA or AA + DHA in the maternal diet at two and five weeks of age. No gross differences in fertility were observed among diet groups at both ages.

2. Fatty Acid Composition of Stomach Contents

The rat pup stomach contents at day 14 of life contained no particulates indicative of diet consumption, and therefore reflected the composition of their dams' milk. The fatty acid composition of stomach contents of rats at two and five weeks of age was analyzed. This analysis reflected dams' milk fatty acid composition (Nouvelot et al., 1983; Lien et al., 1994; Jumpsen et al., 1997a & 1997b) and indicated that the range of dietary fat composition fed in the present experiment produced similar changes in the fat composition of the dams'milk. The major fatty acids in the stomach contents of rats fed either the low LNA, control, or AA + DHA diet treatments at two and five weeks of age were C12:0, C14:0, C16:0, C18:0, C18:1n-7+n-9, and C18:2n-6 (Table 6.2). The C14:0 content of the stomach contents was higher at two compared to five weeks of age (Table 6.2).

Feeding mothers a low LNA compared with the control or AA + DHA diet produced a significant increase in C18:2n-6 and C22:5n-6 content and a decrease in C18:3n-3 content in the stomach at two weeks of age (Table 6.2). Feeding mothers AA + DHA versus control or low LNA diets increased the C22:6n-3 content in the stomach of the rat pups at two weeks of age (Table 6.2). Rat pups fed the low LNA diet showed negligible levels of C22:6n-3 in the stomach at two weeks of age (Table 6.2).

At five weeks of age, feeding rats a low LNA diet also resulted in significantly increased C18:2n-6 content in the stomach compared to animals fed the control or AA + DHA diet (Table 6.2). The C18:3n-3 content in the stomach was lower in animals fed the low LNA diet versus animals fed the control or AA + DHA diet at five weeks of age. The C20:4n-6 and C22:6n-3 content was significantly increased in the stomach of animals fed AA + DHA compared to feeding the control or low LNA diet at five weeks of age. Feeding rats a low LNA compared to feeding rats a control or AA + DHA diet increased the C22:5n-6 content of the rat pups stomach at five weeks of age.

Table 6.2 Content of Fatty Acids in the Stomach of Rats at Two and Five Weeks of Age*

Diet Treatment:	2 Weeks Low LNA^{T}	2 Weeks Control†	2 Weeks AA+DHA‡	5 Weeks Low LNA^{T}	5 Weeks Control†	5 Weeks AA+DHA‡
Fatty acid (% wt/wt)						
C12:0	1.74 ± 0.4^{b}	14.7 ± 0.7^{a}	16.1 ± 1.1^{a}	1.59 ± 0.4^{c}	18.1 ± 3.1^{a}	11.9 ± 0.4^{b}
C14:0	$6.52\pm0.1^{b\surd}$	$9.27\pm0.4^{a\surd}$	$9.54\pm0.7^{a\surd}$	1.20 ± 0.1^{c}	8.26 ± 0.6^{a}	6.71 ± 0.1^{b}
C16:0	9.12 ± 0.0^{b}	17.1 ± 0.4^{a}	17.2 ± 0.1^{a}	11.5 ± 0.1^{b}	19.7 ± 1.9^{b}	14.8 ± 0.2^{a}
C18:0	2.20 ± 0.0^{b}	4.28 ± 0.5^{a}	4.41 ± 0.2^{a}	2.04 ± 0.1^{c}	3.08 ± 0.7^{b}	6.72 ± 0.1^{a}
C18:1n-7 + n-9	11.9 ± 0.1^{c}	$35.8.\pm1.3^{a}$	34.1 ± 1.5^{a}	10.9 ± 0.3^{c}	32.8 ± 1.1^{b}	36.1 ± 0.7^{a}
C18:2n-6	66.9 ± 0.3^{a}	16.2 ± 0.3^{b}	15.3 ± 0.2^{b}	71.0 ± 1.0^{a}	15.7 ± 0.5^{b}	17.8 ± 0.2^{b}
C18:3n-3	0.04 ± 0.0^{b}	1.76 ± 0.0^{a}	1.50 ± 0.2^{a}	0.26 ± 0.0^{c}	1.89 ± 0.2^{b}	2.36 ± 0.0^{a}
C20:4n-6	0.66 ± 0.1^{b}	0.70 ± 0.1^{b}	1.29 ± 0.0^{a}	0.14 ± 0.0^{c}	0.39 ± 0.1^{b}	1.14 ± 0.0^{a}
C20:5n-3	$<0.10^{a}$	0.01 ± 0.0^{a}	0.02 ± 0.0^{a}	0.01 ± 0.0^{a}	$<0.10^{a}$	$<0.10^{a}$
C22:4n-6	$<0.10^{a}$	0.01 ± 0.0^{a}	0.01 ± 0.0^{a}	0.02 ± 0.0^{b}	$<0.10^{b}$	0.87 ± 0.3^{a}
C22:5n-6	1.06 ± 0.0^{a}	0.01 ± 0.0^{b}	0.01 ± 0.0^{b}	1.27 ± 0.7^{a}	$<0.10^{b}$	0.91 ± 0.4^{a}
C22:5n-3	$<0.10^{a}$	$<0.10^{a}$	$<0.10^{a}$	$<0.10^{a}$	$<0.10^{a}$	$<0.10^{a}$
C22:6n-3	$<0.10^{b}$	0.14 ± 0.0^{b}	0.60 ± 0.0^{a}	0.08 ± 0.0^{b}	0.17 ± 0.1^{b}	0.63 ± 0.0^{a}

* Values are mean ± SEM with n=9 for each experimental diet. For each horizontal set of values within an age group that have the same superscript letters (a, b, or c) are not significantly different. Those that have different letters are statistically significantly different, at $p<0.05$.

[T] Low LNA was obtained by the addition of safflower oil as the fat blend.

[†] The control fat diet of C18:2n-6 to C18:3n-3 fatty acid ratio of 7.3:1 approximates the fatty acid composition used in SMA® infant formula.

[‡] The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

[√] Age difference at $p<0.05$.

3. Purity of Synaptic Plasma Membrane Preparations

The purity of the SPM preparation has been described previously (Hargreaves & Clandinin, 1987). The SPM preparation contained only minor cross-contamination (<5%) from microsomes and mitochondria as determined by RNA (SPM, 0.01± 0.0 μg / mg; microsomes, 19.3 ± 0.8 μg / mg) and succinate dehydrogenase (p-iodonitrotetrazolium violet; INT) (SPM, 30.5 ± 7.3 nmol INT reduced / mg / hr; microsomes, 234.3 ± 54.4 nmol INT reduced / mg / hr) analysis, respectively. The SPM marker, Na, K-ATPase, was approximately 8-fold greater in the SPM (35.8 ± 3.1 μmol Pi / mg / hr) compared to the brain homogenate (4.53 ± 1.4 μmol Pi / mg / hr).

4. Synaptic Plasma Membrane Protein Electrophoresis and Immunoblotting

Immunoblots of pooled SPM protein were probed with the anti-β1 antibody; a band at 55 kDa was identified (Appendix 3). No significant differences in relative abundance of β1-subunit protein were found between rat pups at two and five weeks of age (data not shown). The diet treatments had no significant effect on SPM β1-subunit Na, K-ATPase protein abundance at two and five weeks of age (Appendix 3). The relative abundance of β1-subunit protein for rats fed either the low LNA, control, or AA+DHA diet was 34.6, 31.9, 34.5% for two-week-old and 32.0, 35.2, and 32.8% for five-week-old rat pups, respectively (Appendix 3).

5. Synaptic Plasma Membrane Phospholipid and Cholesterol Content

The total and individual phospholipid and cholesterol content, as well as, the fatty acid composition of SPM phospholipids did not differ between male and female rats at two or five weeks of age (data not shown). Thus, statistical analysis to test subsequent effects of diet treatments combined results from both sexes at each age. The amount of total and individual phospholipids and cholesterol content in SPM of rats fed different diets at each age group are similar to that of Breckenridge et al. (1972) and Foot et al. (1982).

The relative distribution of SPM phospholipids from two to five week-old rats is shown (Table 6.3). At all ages and diet treatments examined, the major SPM phospholipids were PC and EPG (Table 6.3). Other investigators have also found that the major phospholipids in rodent SPM were the PC and EPG (Breckenridge et al., 1972; Sun & Sun, 1974; Foot et al., 1982; Hrboticky et al., 1989). There was no significant change in total and individual phospholipids and cholesterol to phospholipid ratio between diet treatments at two and five weeks of age (Table 6.3).

Table 6.3 Total and Individual Phospholipids and Cholesterol Content in Synaptic Plasma Membranes from Rats Fed Different Fat Diets at Two and Five Weeks of Age[*]

	2 Weeks			**5 Weeks**		
Diet Treatment:	**Low LNA[T]**	**Control[†]**	**AA+DHA[‡]**	**Low LNA[T]**	**Control[†]**	**AA+DHA[‡]**
Total PLs[†]	0.65±0.06	0.87±0.21	0.73±0.07	0.66±0.08	0.62±0.09	1.01±0.27
SM[†]	0.13±0.04	0.10±0.02	0.08±0.02	0.06±0.03	0.06±0.02	0.08±0.02
PC[†]	0.19±0.04	0.38±0.08	0.39±0.05	0.25±0.044	0.25±0.03	0.29±0.19
PS[†]	0.13±0.03	0.14±0.03	0.16±0.04	0.14±0.03	0.10±0.02	0.13±0.02
PI[†]	0.10±0.03	0.19±0.06	0.10±0.03	0.10±0.03	0.05±0.02	0.10±0.05
EPG[†]	0.27±0.10	0.37±0.07	0.32±0.05	0.33±0.12	0.25±0.03	0.31±0.02
Cholesterol / PL[‡]	0.37±0.08	0.61±0.26	0.69±0.18	0.39±0.4	0.61±0.09	0.73±0.18

EPG, ethanolamine glycerophospholipid (PE + ethanolamine plasmalogen); PL, phospholipids; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylinositol

[*] Values are mean ± SEM with n=6 for each experimental diet. [†] µmol/mg protein

[‡] µmol/µmol

[T] Low LNA was obtained by the addition of safflower oil as the fat blend.

[†] The control fat diet of C18:2n-6 to C18:3n-3 fatty acid ratio of 7.3:1 approximates the fatty acid composition used in SMA® infant formula.

[‡] The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

6. Fatty Acid Composition of Synaptic Plasma Membrane Phospholipids

a) Total Phospholipids

Consideration of age- and diet-related changes in this study will be given to the C20:4n-6, C22:5n-6, and C22:6n-3 content of rat SPM phospholipids at two and five weeks of age.

SPM total phospholipids were comprised of mostly saturated (C16:0 and C18:0), C18:1n-9, C20:4n-6, and C22:6n-3 (Table 6.4). Age-related changes occurred in C16:0 and C18:1n-7 content of total phospholipids from two to five weeks of age (Table 6.4). No age-related changes were detected in C20:4n-6 and C22:5n-6, and C22:6n-3 content of total phospholipids from two to five weeks of age (Table 6.4). The C20:4n-6 content of total phospholipids was higher in rat pups fed the AA+DHA diet compared to the control or low LNA diet at five but not two weeks of age (Table 6.4). Generally, diet-specific increase in C22:5n-6 and decrease in C22:6n-3 content was observed in the total phospholipids of rat pups fed the low LNA versus control or AA+DHA diets at two and five weeks of age (Table 6.4).

Table 6.4 Fatty Acid Composition of Total Phospholipids in the SPM of Rats Fed Low LNA, Control, or AA + DHA Diet*

	2 Weeks			5 Weeks		
Diet Treatment:	**Low LNA [T]**	**Control[†]**	**AA+DHA[‡]**	**Low LNA [T]**	**Control[†]**	**AA+DHA[‡]**
Fatty acid (% wt/wt)						
C14:0	0.85 ± 0.8^{a}	0.17 ± 0.0^{a}	0.82 ± 0.7^{a}	1.00 ± 0.6^{a}	1.34 ± 1.3^{a}	0.78 ± 0.3^{a}
C16:0	$45.2\pm6.4^{a\surd}$	$31.7\pm0.5^{a\surd}$	$28.5\pm3.4^{a\surd}$	29.6 ± 1.6^{a}	31.4 ± 0.5^{a}	25.2 ± 3.1^{a}
C18:0	23.3 ± 1.3^{a}	20.7 ± 0.2^{a}	18.6 ± 0.7^{a}	22.3 ± 1.4^{a}	24.2 ± 2.1^{a}	24.7 ± 0.9^{a}
C18:1n-9	9.66 ± 1.1^{a}	11.4 ± 0.0^{a}	11.3 ± 0.3^{a}	12.2 ± 0.4^{a}	11.9 ± 0.2^{a}	10.8 ± 2.8^{a}
C18:1n-7	$2.20\pm0.6^{a\surd}$	$2.73\pm0.1^{a\surd}$	$2.65\pm0.1^{a\surd}$	3.45 ± 0.2^{a}	3.49 ± 0.2^{a}	2.84 ± 0.6^{a}
C18:2n-6	1.10 ± 0.1^{a}	0.80 ± 0.0^{ab}	0.44 ± 0.2^{b}	1.13 ± 0.2^{a}	0.51 ± 0.0^{b}	0.76 ± 0.2^{ab}
C18:3n-3	0.34 ± 0.2^{a}	0.01 ± 0.0^{a}	0.01 ± 0.0^{a}	0.04 ± 0.0^{a}	0.07 ± 0.1^{a}	0.07 ± 0.1^{a}
C20:4n-6	6.72 ± 0.7^{b}	12.7 ± 0.1^{a}	15.0 ± 1.7^{a}	9.39 ± 0.7^{a}	8.68 ± 0.4^{a}	11.6 ± 2.5^{a}
C20:5n-3	0.03 ± 0.0^{a}	$<0.10^{a}$	$<0.10^{a}$	$<0.10^{a}$	0.03 ± 0.0^{a}	0.01 ± 0.0^{a}
C22:4n-6	1.24 ± 0.7^{a}	3.05 ± 0.1^{a}	2.36 ± 1.3^{a}	3.42 ± 0.7^{a}	2.72 ± 0.3^{a}	0.81 ± 0.8^{a}
C22:5n-6	3.05 ± 1.2^{a}	1.71 ± 0.1^{a}	2.52 ± 1.2^{a}	6.13 ± 0.6^{a}	1.15 ± 0.2^{b}	2.72 ± 0.9^{b}
C22:5n-3	0.83 ± 0.8^{a}	0.03 ± 0.0^{a}	0.55 ± 0.4^{a}	$<0.10^{a}$	0.05 ± 0.0^{a}	0.45 ± 0.4^{a}
C22:6n-3	5.46 ± 1.8^{b}	15.0 ± 0.4^{a}	17.2 ± 1.8^{a}	11.4 ± 1.3^{b}	14.5 ± 0.3^{ab}	19.2 ± 2.2^{a}

[*] Values are mean ± SEM with n=6 for each experimental diet. For each horizontal set of values within an age group that have the same superscript letters (a or b) are not significantly different. Values that have different letters are statistically significantly different, at $p<0.05$.

[T] Low LNA was obtained by the addition of safflower oil as the fat blend.

[†] The control fat diet of C18:2n-6 to C18:3n-3 fatty acid ratio of 7.3:1 approximates the fatty acid composition used in SMA® infant formula.

[‡] The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

[√] Age difference at $p< 0.05$.

b) Phosphatidylcholine

The effect of feeding low LNA, control, or AA+DHA diet on the fatty acid composition of rat SPM PC is shown (Table 6.5). C16:0, C18:0, C18:1n-9, and C20:4n-6 were the major fatty acids present in PC (Table 6.5). Decrease in C14:0, C16:0, C18:2n-6, C18:3n-3, and C20:4n-6 content and increase in C18:0 and C22:6n-3 content occurred in PC from two to five weeks of age (Table 6.5). A diet-related decrease was found in C20:4n-6 and C22:6n-3 content in rat pups fed the low LNA compared to the control or AA+DHA diet at five but not two weeks of age (Table 6.5).

c) Ethanolamine Glycerophospholipid

EPG constituted the most highly unsaturated phospholipid class in SPM (Table 6.6). The predominant fatty acids in EPG were C16:0, C18:0, C20:4n-6, and C22:6n-3 (Table 6.6). C18:1n-9 and C18:1n-7 content increased and C18:2n-6, C18:3n-3, C20:4n-6, C20:5n-3 content decreased in EPG from two to five weeks of age (Table 6.6). Decrease in the C22:6n-3 content of EPG was observed in rat pups fed the low LNA compared to control or AA+DHA diet at two and five weeks of age (Table 6.6). Feeding rat pups AA+DHA or the control diet increased the C20:4n-6 content in EPG compared to feeding the low LNA diet at two and five weeks of age (Table 6.6).

Table 6.5 Fatty Acid Composition of Phosphatidylcholine in the SPM of Rats Fed Low LNA, Control, or AA + DHA Diet*

		2 Weeks			5 Weeks	
Diet Treatment:	**Low LNA ^{T}**	**Control†**	**AA+DHA‡**	**Low LNA ^{T}**	**Control†**	**AA+DHA‡**
Fatty acid (% wt/wt)						
C14:0	$0.77\pm0.4^{a\surd}$	$0.45\pm0.1^{a\surd}$	$0.67\pm0.2^{a\surd}$	0.47 ± 0.3^{a}	0.04 ± 0.0^{a}	0.14 ± 0.0^{a}
C16:0	$55.0\pm1.4^{a\surd}$	$50.5\pm0.7^{b\surd}$	$53.9\pm0.9^{a\surd}$	52.6 ± 1.4^{a}	43.9 ± 2.1^{b}	47.0 ± 1.3^{b}
C18:0	$9.61\pm0.5^{a\surd}$	$10.3\pm1.0^{a\surd}$	$8.95\pm0.3^{a\surd}$	12.4 ± 0.3^{a}	13.6 ± 0.5^{a}	12.6 ± 0.4^{a}
C18:1n-9	$14.7\pm0.5^{b\surd}$	$17.4\pm0.4^{a\surd}$	$17.7\pm0.3^{a\surd}$	16.1 ± 0.7^{b}	18.9 ± 0.6^{a}	18.7 ± 0.5^{a}
C18:1n-7	$3.18\pm0.2^{a\surd}$	$3.59\pm0.3^{a\surd}$	$3.97\pm0.1^{a\surd}$	4.92 ± 0.5^{b}	5.91 ± 0.2^{a}	4.98 ± 0.1^{b}
C18:2n-6	$2.03\pm0.3^{a\surd}$	$1.25\pm0.1^{b\surd}$	$0.86\pm0.2^{b\surd}$	1.51 ± 0.2^{a}	0.66 ± 0.0^{b}	0.75 ± 0.0^{b}
C18:3n-3	$0.67\pm0.3^{a\surd}$	$0.24\pm0.1^{ab\surd}$	$<0.10^{b\surd}$	0.04 ± 0.0^{a}	0.02 ± 0.0^{a}	$<0.1^{a}$
C20:4n-6	$8.45\pm0.6^{b\surd}$	$10.6\pm0.4^{a\surd}$	$9.22\pm0.3^{b\surd}$	6.71 ± 0.3^{b}	7.79 ± 0.4^{a}	8.63 ± 0.2^{a}
C20:5n-3	0.19 ± 0.1^{b}	0.47 ± 0.2^{a}	$<0.10^{b}$	0.15 ± 0.1^{b}	0.50 ± 0.2^{a}	$<0.10^{b}$
C22:4n-6	0.75 ± 0.2^{a}	0.42 ± 0.2^{a}	0.19 ± 0.2^{a}	0.26 ± 0.2^{a}	0.51 ± 0.2^{a}	0.17 ± 0.1^{b}
C22:5n-6	0.69 ± 0.1^{a}	0.63 ± 0.1^{a}	0.67 ± 0.1^{a}	1.11 ± 0.2^{a}	1.06 ± 0.4^{a}	0.83 ± 0.1^{a}
C22:5n-3	0.41 ± 0.2^{a}	0.13 ± 0.0^{b}	0.08 ± 0.0^{b}	0.96 ± 0.5^{a}	0.16 ± 0.1^{ab}	0.04 ± 0.0^{b}
C22:6n-3	$3.25\pm0.3^{b\surd}$	$4.31\pm0.3^{a\surd}$	$3.85\pm0.3^{ab\surd}$	2.85 ± 0.2^{b}	7.06 ± 0.4^{a}	6.21 ± 0.2^{a}

* Values are mean ± SEM with n=6 for each experimental diet. For each horizontal set of values within an age group that have the same superscript letters (a or b) are not significantly different. Values that have different letters are statistically significantly different, at $p<0.05$.

^{T} Low LNA was obtained by the addition of safflower oil as the fat blend.

† The control fat diet of C18:2n-6 to C18:3n-3 fatty acid ratio of 7.3:1 approximates the fatty acid composition used in SMA® infant formula.

‡ The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

$^{\surd}$ Age difference at $p<0.05$

Table 6.6 Fatty Acid Composition of Ethanolamine Glycerophospholipid in the SPM of Rats Fed Low LNA, Control, or AA + DHA Diet*

Diet Treatment:	2 Weeks Low LNA[T]	2 Weeks Control[†]	2 Weeks AA+DHA[‡]	5 Weeks Low LNA[T]	5 Weeks Control[†]	5 Weeks AA+DHA[‡]
Fatty acid (% wt/wt)						
C14:0	1.04 ± 0.2^{a}	0.01 ± 0.0^{a}	0.77 ± 0.7^{a}	0.78 ± 0.2^{a}	0.67 ± 0.6^{a}	0.94 ± 0.8^{a}
C16:0	22.5 ± 1.6^{a}	7.47 ± 0.3^{a}	7.32 ± 0.6^{a}	14.0 ± 0.7^{a}	8.89 ± 0.5^{a}	17.1 ± 8.6^{a}
C18:0	29.7 ± 1.7^{a}	29.5 ± 0.6^{a}	28.7 ± 1.3^{a}	30.5 ± 1.2^{b}	37.0 ± 2.3^{a}	25.3 ± 3.0^{b}
C18:1n-9	$5.88\pm0.4^{a\surd}$	$6.73\pm0.3^{a\surd}$	$6.55\pm0.2^{a\surd}$	6.57 ± 0.3^{b}	8.89 ± 0.6^{a}	6.38 ± 0.7^{b}
C18:1n-7	$0.48\pm0.2^{a\surd}$	$0.80\pm0.0^{a\surd}$	$0.86\pm0.0^{a\surd}$	1.04 ± 0.1^{a}	1.23 ± 0.1^{a}	0.88 ± 0.1^{a}
C18:2n-6	$2.36\pm0.5^{a\surd}$	$0.53\pm0.1^{b\surd}$	$0.30\pm0.1^{b\surd}$	0.83 ± 0.1^{a}	0.32 ± 0.0^{b}	0.19 ± 0.0^{b}
C18:3n-3	$0.53\pm0.1^{a\surd}$	$0.33\pm0.1^{a\surd}$	$<0.10^{b\surd}$	0.08 ± 0.1^{a}	$<0.10^{a}$	$<0.10^{a}$
C20:4n-6	$15.3\pm1.3^{b\surd}$	$24.1\pm0.4^{a\surd}$	$24.0\pm0.6^{a\surd}$	14.2 ± 0.9^{a}	16.9 ± 0.2^{a}	15.3 ± 1.7^{a}
C20:5n-3	$1.45\pm0.3^{a\surd}$	$0.05\pm0.0^{b\surd}$	$<0.10^{b\surd}$	0.27 ± 0.1^{a}	$<0.10^{b}$	$<0.10^{b}$
C22:4n-6	0.89 ± 0.3^{a}	0.03 ± 0.0^{a}	2.59 ± 1.3^{a}	1.43 ± 1.4^{a}	1.68 ± 1.1^{a}	0.91 ± 0.9^{a}
C22:5n-6	4.27 ± 0.4^{a}	6.14 ± 0.1^{a}	3.71 ± 1.1^{a}	7.89 ± 0.4^{a}	2.58 ± 0.5^{a}	3.35 ± 1.0^{a}
C22:5n-3	2.80 ± 0.3^{a}	1.56 ± 0.0^{b}	1.06 ± 0.3^{b}	4.74 ± 0.3^{a}	0.34 ± 0.1^{b}	0.98 ± 0.2^{b}
C22:6n-3	12.8 ± 1.4^{b}	22.7 ± 0.4^{a}	24.2 ± 1.0^{a}	17.7 ± 1.0^{b}	21.5 ± 1.9^{b}	28.7 ± 3.1^{a}

* Values are mean ± SEM with n=6 for each experimental diet. For each horizontal set of values within an age group that have the same superscript letters (a or b) are not significantly different. Values that have different letters are statistically significantly different, at $p<0.05$.

[T] Low LNA was obtained by the addition of safflower oil as the fat blend.

[†] The control fat diet of C18:2n-6 to C18:3n-3 fatty acid ratio of 7.3:1 approximates the fatty acid composition used in SMA® infant formula.

[‡] The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

[√] Age difference at $p<0.05$

d) Phosphatidylserine

The major fatty acids of SPM PS were C16:0, C18:0, C18:1n-9, C20:4n-6, C22:4n-6, C22:5n-6, and C22:6n-3 (Table 6.7). PS contained high levels of C22:6n-3 (Table 6.7). C18:0 and C18:3n-3 content of PS was higher at two compared to five weeks of age (Table 6.7). The C18:1n-9, C22:5n-6, and C22:6n-3 content of PS was generally higher at five compared to two weeks of age. A diet-associated decrease in C22:6n-3 along with increase in C22:5n-6 occurred in rats fed the low LNA versus rats fed the control or AA+ DHA diet at two and five weeks of age (Table 6.7). Rat pups fed the AA+DHA diet at five weeks of age contained a significantly higher C20:4n-6 content in PS compared to animals fed the low LNA or control diet (Table 6.7).

e) Phosphatidylinositol

The PI fraction from SPM contained a large amount of saturated fatty acids, particularly, C16:0 and C18:0 (Table 6.8). C20:4n-6 and C22:6n-3 present in PI were the predominant n-6 and n-3 fatty acids, respectively. Increase in C20:4n-6 content of PI occurred in rat pups from two to five weeks of age (Table 6.8). The C20:4n-6 and C22:6n-3 content of PI was lower in rats fed the low LNA compared to rats fed the control or AA + DHA diet at five weeks of age (Table 6.8).

Table 6.7 Fatty Acid Composition of Phosphatidylserine in the SPM of Rats Fed Low LNA, Control, or AA + DHA Diet[*]

	2 Weeks			5 Weeks		
Diet Treatment:	**Low LNA[T]**	**Control[†]**	**AA+DHA[‡]**	**Low LNA[T]**	**Control[†]**	**AA+DHA[‡]**
Fatty acid (% wt/wt)						
C14:0	0.57±0.2^{a}	0.16±0.1^{a}	0.18±0.1^{a}	0.54±0.2^{a}	0.05±0.0^{b}	0.05±0.0^{b}
C16:0	12.9±2.0^{a}	8.01±1.3^{a}	12.6±2.2^{a}	15.6±1.0^{a}	3.94±0.3^{c}	6.21±0.5^{b}
C18:0	30.0±2.6$^{a\surd}$	37.9±1.9$^{a\surd}$	34.0±2.6$^{a\surd}$	32.7±2.1^{b}	37.7±0.3^{a}	15.1±0.3^{c}
C18:1n-9	6.46±0.7$^{b\surd}$	8.62±1.0$^{a\surd}$	8.28±0.6$^{ab\surd}$	9.37±0.4^{b}	8.98±0.2^{b}	11.4±0.1^{a}
C18:1n-7	1.02±0.3^{a}	0.71±0.1^{a}	1.29±0.3^{a}	0.95±0.3^{a}	0.53±0.0^{a}	0.99±0.1^{a}
C18:2n-6	1.85±0.6^{a}	0.90±0.7^{a}	0.38±0.0^{a}	0.88±0.2^{a}	0.23±0.1^{a}	0.31±0.1^{a}
C18:3n-3	4.33±1.4$^{a\surd}$	1.79±1.1$^{ab\surd}$	0.42±0.4$^{b\surd}$	0.03±0.0^{a}	<0.10^{a}	0.08±0.0^{a}
C20:4n-6	8.16±1.4^{a}	9.28±1.4^{a}	10.0±0.4^{a}	3.81±0.7^{b}	2.81±0.4^{b}	16.7±0.2^{a}
C20:5n-3	4.14±1.1$^{a\surd}$	0.04±0.0$^{b\surd}$	0.14±0.1$^{b\surd}$	0.39±0.1^{a}	0.15±0.0^{b}	<0.10^{b}
C22:4n-6	6.12±1.1^{a}	5.15±0.9^{a}	5.78±0.3^{a}	5.13±0.2^{b}	0.11±0.0^{c}	8.71±0.1^{a}
C22:5n-6	4.60±0.3$^{a\surd}$	1.89±0.3$^{b\surd}$	1.34±0.4$^{b\surd}$	10.0±0.5^{a}	4.70±0.2^{b}	0.86±0.1^{c}
C22:5n-3	2.21±0.7^{a}	1.54±1.2^{a}	0.30±0.1^{a}	<0.10^{c}	2.25±0.1^{a}	0.33±0.0^{b}
C22:6n-3	17.7±2.1$^{b\surd}$	24.0±2.5$^{a\surd}$	25.2±0.9$^{a\surd}$	20.6±1.0^{b}	38.6±0.6^{a}	39.2±0.4^{a}

[*] Values are mean ± SEM with n=6 for each experimental diet. For each horizontal set of values within an age group that have the same superscript letters (a or b) are not significantly different. Values that have different letters are statistically significantly different, at $p<0.05$.

[T] Low LNA was obtained by the addition of safflower oil as the fat blend.

[†] The control fat diet of C18:2n-6 to C18:3n-3 fatty acid ratio of 7.3:1 approximates the fatty acid composition used in SMA® infant formula.

[‡] The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

[√] Age difference at $p<0.05$

Table 6.8 Fatty Acid Composition of Phosphatidylinositol in the SPM of Rats Fed Low LNA, Control, or AA + DHA Diet*

		2 Weeks			5 Weeks	
Diet Treatment:	**Low LNA ^{T}**	**Control†**	**AA+DHA‡**	**Low LNA ^{T}**	**Control†**	**AA+DHA‡**
Fatty acid (% wt/wt)						
C14:0	1.74±0.5^{a}	0.35±0.1^{b}	0.98±0.4^{ab}	2.36±0.6^{a}	0.30±0.2^{b}	0.88±0.6^{ab}
C16:0	29.3±5.6^{a}	14.4±6.8^{b}	18.4±2.3^{b}	24.8±3.3^{a}	14.5±2.6^{b}	12.8±2.3^{b}
C18:0	27.9±1.7^{b}	34.6±1.7^{a}	36.5±1.8^{a}	30.1±2.7^{a}	31.3±2.5^{a}	33.0±1.1^{a}
C18:1n-9	6.85±1.0^{a}	8.42±0.5^{a}	5.26±1.5^{a}	7.64±1.8^{a}	7.65±1.5^{a}	7.45±1.2^{a}
C18:1n-7	0.61±0.3^{a}	1.51±0.2^{a}	2.18±0.2^{a}	1.46±0.5^{a}	1.72±0.2^{a}	1.49±0.2^{a}
C18:2n-6	2.47±0.9^{a}	0.69±1.6^{b}	0.54±0.0^{b}	1.75±0.4^{a}	0.59±0.1^{b}	0.68±0.1^{b}
C18:3n-3	1.26±0.4^{a}	1.69±0.5^{a}	4.01±1.7^{a}	2.87±1.8^{a}	0.11±0.5^{a}	0.33±0.3^{a}
C20:4n-6	21.2±4.4$^{a\surd}$	23.5±3.1$^{a\surd}$	22.8±2.2$^{a\surd}$	20.8±1.9^{b}	33.1±3.0^{a}	31.6±4.2^{a}
C20:5n-3	0.75±0.3^{a}	0.87±0.3^{a}	0.70±0.6^{a}	0.97±0.4^{a}	0.85±0.8^{a}	0.67±0.6^{a}
C22:4n-6	2.19±0.9^{a}	2.39±0.6^{a}	2.18±0.7^{a}	1.80±0.6^{a}	1.65±1.0^{a}	1.95±0.9^{a}
C22:5n-6	1.39±0.6^{a}	0.74±0.2^{a}	0.23±0.1^{a}	1.44±0.5^{a}	0.70±0.2^{a}	0.65±0.1^{a}
C22:5n-3	0.13±0.1^{a}	0.31±0.1^{a}	0.38±0.2^{a}	0.26±0.1^{a}	<0.10^{a}	0.01±0.0^{a}
C22:6n-3	4.20±0.8^{b}	10.6±1.4^{a}	5.87±0.5^{b}	3.71±0.7^{b}	7.55±1.4^{a}	8.49±1.5^{a}

* Values are mean ± SEM with n=6 for each experimental diet. For each horizontal set of values within an age group that have the same superscript letters (a or b) are not significantly different. Values that have different letters are statistically significantly different, at $p<0.05$.

^{T} Low LNA was obtained by the addition of safflower oil as the fat blend.

† The control fat diet of C18:2n-6 to C18:3n-3 fatty acid ratio of 7.3:1 approximates the fatty acid composition used in SMA® infant formula.

‡ The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

$^{\surd}$ Age difference at $p<0.05$

7. Na, K-ATPase Kinetics

Kinetic parameters of Na, K-ATPase, obtained from SPM of two and five week-old rats, were examined and the results are presented as Eadie-Hofstee plots (Figure 6.1). Among the three diet treatments, rat pups at two weeks of age showed a low activity (Vmax = 7.85 – 21.8 μmol Pi/ mg / hr) and high affinity (Km = 0.49 – 1.04 mM) compared to rat pups at five weeks of age which showed a high activity (Vmax = 24.92 – 45.33 μmol Pi/ mg / hr) and low affinity (Km = 1.56 – 2.44 mM) (Table 6.9).

The Na, K-ATPase kinetics in the SPM of rats fed either the low LNA, control, or AA+DHA diets are shown (Figure 6.1). Rat pups fed the low LNA diet showed a decrease in Vmax compared to pups consuming the control or AA+DHA diet at two weeks of age (Figure 6.1). The Vmax of Na, K-ATPase at two weeks of age in low LNA rats was 7.85 compared to 21.6 and 21.8 μmol Pi/ mg / hr for control or AA + DHA diet, respectively (Table 6.9). The Vmax of Na, K-ATPase at five weeks of age in rats fed low LNA was 24.9 compared to 45.3 and 39.7 μmol Pi/ mg / hr for rats fed control or AA + DHA diet, respectively.

The Km of Na, K-ATPase in SPM of rat pups at both ages also showed differences among diet treatments. The Km of Na, K-ATPase in SPM of rat pups fed the low LNA or control diet was lower compared to the rats fed AA+DHA diet at two weeks of age. The Km of Na, K-ATPase at two weeks of age was 0.57, 0.49, and 1.04 mM for rats fed the low LNA, control, or AA+DHA diet, respectively. Rat pups fed the low LNA diet had a higher Km compared to the rats fed the control or AA+DHA diet at five weeks of age. The Km of the SPM Na, K-ATPase of rats fed the low LNA was 2.44 mM compared to 1.56 and 1.67 mM for the rats fed control or AA+DHA diet, respectively.

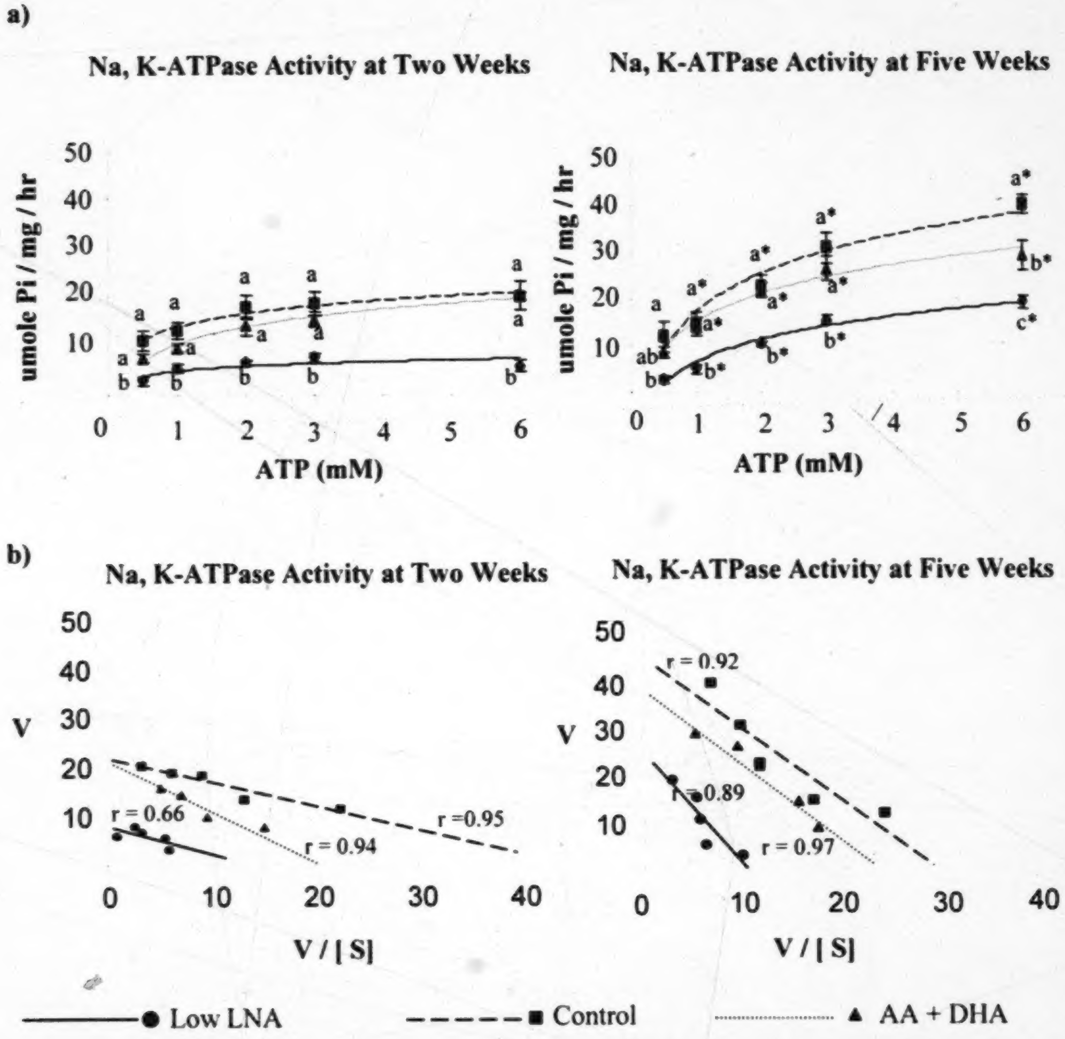

Figure 6.1 Synaptic Plasma Membrane Na, K-ATPase Kinetics of Rats Fed Low LNA, Control, or AA + DHA Diet at Two and Five Weeks of Age. Specific activity was determined by subtracting the activity observed in the presence of 1 mM ouabain from total activity. a) a Michaelis-Menten plot of Na, K-ATPase activity (μmol Pi/ mg / hr; V) vs. ATP (mM). Each point represents mean ± SEM of six experiments performed in triplicates. Values without a common letter among diet treatments at each ATP concentration (mM) differ significantly ($p < 0.05$). *Age differences at each ATP concentration (mM) at $p < 0.05$; b) an Eadie-Hofstee plot of Na, K-ATPase activity (V) vs. Na, K-ATPase (V) / ATP (mM) [S]. The correlation coefficient (r) for straight line in the graph are indicated (df=4). Values are mean with n=6 for each experimental diet.

D. DISCUSSION

The present study was conducted to compare the effects of feeding rat pups diets with C18:3n-3 vs. C22:6n-3 on the SPM lipid composition and kinetic properties of Na, K-ATPase at two and five weeks of age. The results of this study revealed that rat pups fed a low LNA compared to animals fed the control or C22:6n-3 diet have increased SPM phospholipid C22:5n-6 content, decreased SPM phospholipid C22:6n-3 and C20:4n-6 content and decreased SPM Na, K-ATPase activity at two and five weeks of age.

Age- and Diet-Related Changes in Synaptic Plasma Membrane Phospholipid and Cholesterol Content

The developmental changes in rat SPM lipid composition during the first five weeks of life were small (Table 6.3). The lack of developmental change in rat SPM total and individual phospholipid and cholesterol to phospholipid ratio during the first five weeks of life is similar to that of weanling rats (Foot et al., 1982; Hofteig et al., 1985) and piglets (Hrboticky et al., 1989).

Dietary fat had a non-significant effect on SPM PC content (Table 6.3). The reason for the higher PC content in rats fed control or AA + DHA diet compared to the low LNA diet at two weeks of age is unknown. However, Hitzemann (1982) found that PEMT activity was highest in rat synaptosomes at two weeks of age. It is possible that the higher PC content in rats fed control or AA + DHA diet compared to feeding rats a low LNA diet may be due to the increased PEMT activity at two-weeks of age.

The content of PS in SPM of rats was not significantly affected by the diet fat treatments used in this study (Table 6.3). Ikemoto et al (2000) which showed that dietary C18:3n-3 deficiency does not affect PS synthesis in rat synaptosomes.

The rats fed the control or AA + DHA diet showed a higher cholesterol to phospholipid ratio compared to rats fed the low LNA diet at two and five weeks of age. The higher cholesterol to phospholipid ratio in SPM of rats fed control or AA + DHA diet at two and five weeks of age may compensate for the higher n-3 fatty acids, particularly, the C22:6n-3 content in total phospholipids (Table 6.4).

Age- and Diet-Related Changes in Synaptic Plasma Membrane Phospholipid Fatty Acid Composition

The high levels of C20:4n-6 and C22:6n-3 in rat SPM phospholipids in this study are similar to data published by Breckenridge et al. (1972), Sun & Sun (1974), Foot et al. (1982), and Hargreaves et al. (1989). The developmental changes observed in rat SPM phospholipids C16:0 and C18:0 and the general increase in C22:6n-3 and decrease in C20:4n-6 fatty acid, which were observed in total phospholipids, PC, PS, and EPG are similar to data from other rodent SPM (Foot et al., 1982).

The major effect of feeding the low LNA compared to control or AA + DHA diet on the rat SPM was depletion of C22:6n-3 and a reciprocal enrichment of C22:5n-6. Similar changes have been reported for SPM in rodents fed diets containing high C18:2n-6:C18:3n-3 fatty acid ratio (Foot et al., 1982; Bourre et al., 1984). Conceivably, this effect may be explained by competition of n-6 and n-3 fatty acids for microsomal desaturation (Brenner & Peluffo, 1966; Cook, 1978; Sanders & Rana, 1987). Therefore, the significantly lower C22:6n-3 content in SPM phospholipids of rats fed the low LNA diet was compensated by a significantly higher level of docosapentaenoic acid (C22:5n-6), indicating that polyunsaturation of membrane phospholipids was preserved in agreement with previous reports (Youyou et al., 1986; Connor et al., 1990).

The higher C20:4n-6 and C22:6n-3 content in SPM phospholipids of rats fed the AA + DHA diet compared to control or low LNA diet is similar to data by Anderson et al. (1990) and Jumpsen et al. (1997) which demonstrated that preformed C20:4n-6 and C22:6n-3 are more readily incorporated into brain phospholipids. Both delta-6- and delta-5 desaturases are present in liver and brain of the newborn rodents (Ravel et al., 1985; Sanders & Rana, 1987; Bourre et al., 1990) and some C18:2n-6 and C18:3n-3 can be converted to C20:4n-6 and C22:6n-3, respectively, in the suckling rat (Nouvelot et al., 1983). The data from this study demonstrates that the newborn rat is able to convert C18:2n-6 and C18:3n-3 into C20:4n-6 and C22:6n-3, respectively, but it is not as effective as preformed C20:4n-6 and C22:6n-3 at raising the SPM phospholipid C20:4n-6 and C22:6n-3 content.

Age- and Diet-Related Changes in Synaptic Plasma Membrane Na, K-ATPase Kinetics

The kinetic parameters of Na, K-ATPase obtained from the present study shows that two-week-old relative to five-week-old rat pups possess high affinity ATP binding sites and low activity of SPM Na, K-ATPase. Conversely, the five-week-old relative to two-week-old rat pups have low affinity ATP binding sites and high activity SPM Na, K-ATPase. The results of this study are in agreement with those of Samson & Quin (1967), Mishra & Shankar (1980), and Tsutsumi et al. (1995), showing enhanced Na, K-ATPase activity with age. These findings of increased SPM Na, K-ATPase activity with age differ from those reported by Calderini et al. (1983) who showed a decrease (≈ 35%) in SPM Na, K-ATPase with age. The reason for this discrepancy between studies may be due to the time periods in which Na, K-ATPase activity was measured (weeks vs. months). The reason for this shift in Na, K-ATPase kinetics may be due to a mechanism essential for maintenance of ionic gradients at very low concentration of ATP (Tsutsumi et al., 1995). Expression of different molecular forms of Na, K-ATPase could account for the altered Na, K-ATPase kinetics with age (Tsutsumi et al., 1995). Thus, a high Na, K-ATPase affinity at a young age permits a greater tolerance of low levels of ATP (Tsutsumi et al., 1995).

The results of the present study show no significant changes in the total and individual change in the total and individual SPM phospholipid content between age and diet treatments. This finding is important because it has been shown that membrane phospholipids, such as PC (Tanaka & Strickland, 1965), PS (Tsakris & Deliconstantinos, 1984), and PE (Goldman & Albers, 1973) have a significant effect on Na, K-ATPase activity. Therefore, the lower Vmax of SPM Na, K-ATPase of rats fed low LNA compared to rats fed control or AA + DHA diet is not attributed to altered phospholipid content.

It is well known that alterations in the lipid environment of membrane-bound enzymes may be associated with changes in kinetics parameters such as Vmax and Km for substrate (reviewed by Lenaz, 1979). The low Vmax of Na, K-ATPase in SPM of rats fed low LNA compared to rats fed control or AA + DHA diet is similar to that reported by Bourre et al. (1989) and Tsutsumi et al. (1995). The increased C22:5n-6 and decreased

C22:6n-3 in the low LNA compared to control or AA + DHA fed rats may account for the low Vmax in low LNA compared to control or AA + DHA fed rats at two and five weeks of age. Recently, Swarts et al. (1990) showed a decrease in the dephosphorylation rate of the phosphorylated intermediate of Na, K-ATPase in the presence of unsaturated fatty acids, particularly n-6 fatty acids. Therefore the increase in C22:5n-6 content in SPM phospholipids of rats fed the low LNA diet could result in lower levels of the dephosphorylated form of Na, K-ATPase which may decrease Na, K-ATPase activity because dephosphorylated form of Na, K-ATPase is needed to bind potassium.

A role of dietary fat on Na, K-ATPase gene or protein expression could potentially be responsible for the differences in Na, K-ATPase Vmax for rat pups fed either the low LNA, control, or AA + DHA diet (Takeuchi et al., 1995; Clarke & Jump, 1996). Western blot analysis of the β1-subunit of SPM Na, K-ATPase did not show any significant difference in amount of Na, K-ATPase β1-subunit protein among diet groups at two and five weeks of age (data stated in results section). This finding is in agreement with Sato et al. (1999) which showed that long-term C18:3n-3 deficiency induces no substantial change in the rate of protein synthesis in rat brain. Therefore, the significant increase in Na, K-ATPase Vmax for rat pups fed either control or AA + DHA compared to the pups fed the low LNA diet is attributed to alterations in the SPM lipid environment.

The physiological significance of a lower Na, K-ATPase activity in animals fed the low LNA compared to the control or AA + DHA diet is unknown. Inhibition of SPM Na, K-ATPase activity may result in decreased reuptake as well as increased release of neurotransmitters (Benzi et al.,1993). Conceivably, the altered learning and memory abilities in rats and mice fed the low LNA diet may be due to lower Na, K-ATPase activity which may be a biochemical basis for the altered brain functions (Lamptey & Walker, 1976; Yamamoto et al., 1987; Bourre et al., 1989; Yonekubo et al., 1994; Frances et al., 1996; Belzung et al., 1998; Carrie et al., 1999 & 2000).

In summary, this study demonstrates that developmental changes in rat SPM lipids from two to five weeks of age are not significantly altered by the diet fat treatments used in this study. Feeding rats diets with either C18:3n-3 or C22:6n-3 alters the SPM phospholipid fatty acid content, particularly, C20:4n-6, C22:5n-6, and C22:6n-3. The subsequent alterations in SPM Na, K-ATPase kinetic properties suggests that SPM phospholipid C20:4n-6, C22:5n-6, and C22:6n-3 content plays an important role in modulating Na, K-ATPase activity. Thus, the findings from the present study may have important implications for infant formulas that are devoid of C22:6n-3.

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CHAPTER VII. DIETARY N-6 AND N-3 FATTY ACIDS ALTERS SYNAPTIC PLASMA MEMBRANE GANGLIOSIDE FATTY ACID COMPOSITION OF RATS AT TWO WEEKS OF AGE

A. INTRODUCTION

Gangliosides (sialic acid containing glycosphingolipids) are plasma membrane constituents, which are especially abundant in neuronal tissues (Panzetta & Allende, 2000). Although their precise function has not been clearly defined, gangliosides do play an important role in cell-cell interaction (Cheresh et al., 1986; Baker, 1988; Yang et al., 1996), recognition and signaling (Bremer et al., 1986; Hakomori, 1990; Nagai, 1995; Meuillet et al., 1996), neuritogenesis (Wu et al., 1995), and cellular immunity (Massa, 1993; Lu & Sharom, 1995). It has been shown using artificial (Palestini et al., 1994; Stewart & Boggs, 1993) and intact biological membranes (Palestini et al., 1991) that the hydrophobic portion (ceramide) of glycosphingolipids is involved in controlling the availability of gangliosides to interact with external ligands and enzymes as well as governing the aggregative and lateral phase separation properties (Masserini & Freire, 1986; Masserini et al., 1989; Palestini et al., 1991).

Previous studies have shown that ganglioside pattern and ceramide composition can undergo substantial changes with age (Merat & Dickerson, 1973; Ohsawa, 1989; Palestini et al., 1990 & 1991). In this regard, in rat brain, it was shown that the long-chain base component of ceramides containing 18 and 20 carbon atoms and one double bond (C18:1 and C20:1) constitute up to 97% of total long-chain base content at all ages (Palestini et al., 1990 & 1991). The C20:1 long-chain base occurs only in postnatal life and increases with age in all gangliosides with a maximal content in aged animals (Palestini et al., 1990 & 1991). The fatty acid composition of the ceramide moiety from the brain gangliosides tends to remain constant with age with stearic acid (C18:0) being the predominant fatty acid species (Palestini et al., 1990 & 1991).

Although there are extensive studies of developmental changes of brain gangliosides, studies concerning the effect of dietary fat on brain ganglioside fatty acid composition during development are limited. Berra et al. (1976) examined the effects of diets with different fatty acid composition on whole brain gangliosides of rats from birth to 100 days of age. The authors reported that the fatty acid composition of whole brain ganglioside was unaffected by the diet treatments. However, the authors did not show any data on the fatty acid composition of the gangliosides and the fat content of the diet treatments was different. Recently, Saito & Saito (1991) reported that C22:0 and C24:1 added to cell culture media can be incorporated into the fatty acyl moiety of SM and gangliosides of cultured neuronal cells. Studies with neurotumor cell lines have shown incorporation of ^{3}H –C20:4n-6 into globosides and gangliosides (Dawson & Vartanian, 1988). Taken together, these studies demonstrate that the fatty acid composition of gangliosides can be altered and that PUFAs can be detected as part of the fatty acyl moiety of gangliosides. It is well established that C20:4n-6 and C22:6n-3 are present in membrane phospholipids, however, it is not known if C20:4n-6 and C22:6n-3 are present in SPM gangliosides.

Since gangliosides are enriched in SPM and modulation of some membrane functions may depend on the ceramide moiety of gangliosides, it is conceivable that dietary fat induced changes in the fatty acid composition of the ceramide moiety of brain gangliosides may affect membrane function similar to that shown with membrane phospholipids (reviewed by Stubbs & Smith, 1984 and Clandinin et al., 1997). Therefore, the objectives of this study were to determine if C20:4n-6 and C22:6n-3 are present in rat SPM gangliosides and to investigate whether dietary fat can alter the C20:4n-6 and C22:6n-3 content of rat SPM gangliosides.

B. MATERIALS AND METHODS

1. Animal Care

Breeding of Sprague-Dawley rats have been described in **Chapter III**. All litters were culled to twelve rat pups following parturition. Rats sacrificed at two weeks of age received only maternal milk. One entire litter of rat pups fed the same diet was sexed and weighed before decapitation. Excised brains were placed in ice-cold 0.32 M sucrose with 1mM EDTA, pepstatin A (20μg/mL), aprotinin (20 μg/mL), trypsin inhibitor (20 μg/mL), phenylmethylsulfonyl fluoride (5 μg/mL), and leupeptin (20μg/mL), pH 7.4. Thirty-six brains were pooled per sample for isolation of SPM. Thirty-six brains were determined to be the minimum amount needed to detect the fatty acid composition of SPM gangliosides. Stomach contents of three rats from each litter were also removed and analyzed to determine the fatty acid composition of maternal milk. Three litters per diet treatment were used.

2. Diets

Three semi-synthetic diets were used as described in **Chapter VI** (Table 6.1). To minimize fatty acid peroxidation, the diets were sealed under nitrogen and stored in a freezer at -30°C in darkness. Each day the required amount of diet was taken out, mixed thoroughly, and placed in individual feed cups.

3. Isolation of Synaptic Plasma Membrane

SPMs from rat pups at two of age were isolated as described in **Chapter VI**.

4. Lipid Analysis

Total gangliosides were extracted from rat SPM according to the method of Folch (1957), which involves SPM extraction with chloroform:methanol (2:1, by vol.). Partitioning of gangliosides was carried out using 0.1 M KCl. The ganglioside containing aqueous phase was hydrolyzed with 5 mL of 0.5 M potassium hydroxide in methanol: water (1:1, by vol.) at room temperature overnight to remove any glycerophospholipids present (Mansson et al, 1978). The pH of the solution was adjusted to 5-6 with HCl and methanol was removed from the solution by a rotary evaporator (Mansson et al, 1978). The remaining aqueous solution was lyophilized and resuspended in 10 mL 0.1 M KCl. This solution was applied three times to a Sep-Pak™ reverse-phase C18 cartridge (Waters Associates) and gangliosides were eluted with chloroform : methanol (2:1, by vol.) and methanol (Williams & McCluer, 1980). Fractions containing gangliosides were evaporated to dryness under nitrogen. The dry residue was dissolved in chloroform : methanol (2:1, by vol.) and stored at -30°C until analysis.

Identification of individual gangliosides was performed by high-performance thin-layer chromatography (HPTLC) using 10 x 10 cm silica gel 60 HPTLC plates (Whatman, Clifton, NJ). After spotting the sample on the HPTLC plate, the plate was developed with chloroform : methanol (85:15, by vol.) to remove interfering neutral lipids and phospholipids (Yu et al., 1988). When the plates were completely air-dried, the individual gangliosides were separated by developing the HPTLC plate in chloroform : methanol : 0.02% (wt/vol.) $CaCl_2 \cdot 2H_2O$ (55:45:10, by vol.; Ando et al., 1978; Appendix 3).

To confirm the presence of gangliosides in the SPM, some SPM ganglioside samples were visualized on the HPTLC plate with resorcinol reagent spray (Svennerholm et al., 1957) and characterized by comparison with standard bovine brain gangliosides (GM1, GD1a, GD1b, and GT1b; Sigma Chemical Co., St. Louis, MO). For fatty acid analysis of SPM gangliosides, the separated gangliosides on the HPTLC plate were sprayed with 0.01% (wt/vol.) primulin in water and visualized under UV light. Ganglioside fractions on the HPTLC plate corresponding to standards were scraped into culture tubes. Fatty acid methyl esters were prepared with 1.0 M hydrochloric acid in methanol for 26 hr at 80°C (Mansson et al., 1978).

5. Fatty Acid Analysis

Fatty acid methyl esters were analyzed by automated gas-liquid chromatography as described in **Chapter III**.

6. Gas-Chromatography Mass Spectrometry

For final identification of fatty acids in SPM gangliosides, a Hewlett Packard 5890 GLC equipped with a flame ionization detector MS ChemStation (HP-UX series) data system was used. Mass spectra of individual saturated, monounsaturated, and polyunsaturated fatty acid in SPM gangliosides were compared with mass spectra of the corresponding standards.

C. RESULTS

1. Growth Characteristics

The body and brain weights of rat pups at two weeks of age are similar to that shown in **Chapter VI.**

2. Fatty Acid Composition of Stomach Content

The rat pup stomach contents at day 14 of life contained no particulates indicative of diet consumption, and therefore reflected the composition of their dams' milk. The fatty acid composition of the stomach contents of rat pups fed low LNA, control, or AA+DHA diet at two weeks of age was similar to that shown in **Chapter VI** (Table 6.2).

3. Purity of SPM Preparations

The SPM purity was tested as described in **Chapter VI**.

4. Gas-Chromatography Mass Spectrometry Analysis

Mass spectra of C20:4n-6 and C22:6n-3 from SPM gangliosides were identical with mass spectra of corresponding standards. These data appear to be the first evidence to show the presence of C20:4n-6 and C22:6n-3 in rat SPM gangliosides (Figure 7.1).

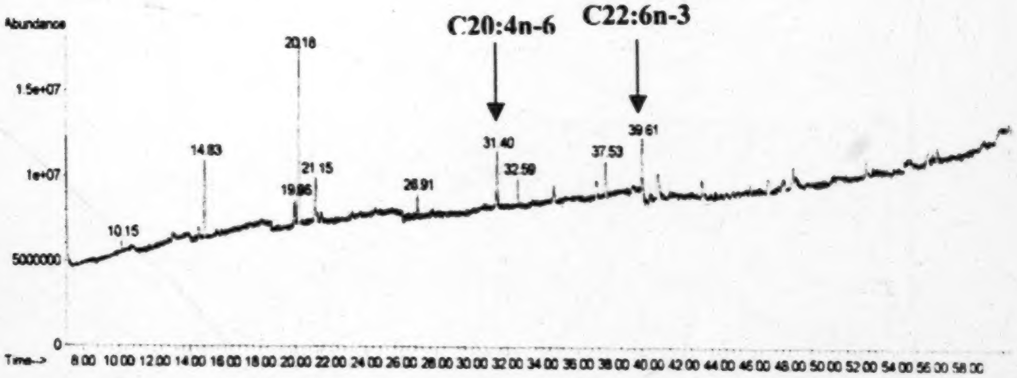

Figure 7.1 Total Ion Chromatogram of C20:4n-6 and C22:6n-3 methyl esters from GM1a in Synaptic Plasma Membrane of Rats at Two Weeks of Age

5. Dietary Fat Changes in SPM Ganglioside Fatty Acid Content

Fatty acid composition of GM1a, GD1a, GD1b, and GT1b from the rat SPM at two-weeks of age are shown in Table 7.1, Table 7.2, Table 7.3, and Table 7.4, respectively. The most abundant molecular species present in the SPM individual ganglioside fractions were C16:0 and C18:0, a feature already reported for gangliosides from whole brain (Palestini et al., 1990). The diet treatments used in this study appeared to alter the fatty acid composition of the individual gangliosides differently. The proportion of C16:0 was higher and C18:0 lower in GM1a and GD1a (Table 7.1 and 7.2) compared to GD1b and GT1b (Table 7.3 and 7.4) with the three diet treatments. The C20:4n-6 and C22:6n-3 content of rat SPM gangliosides was also altered by the diet treatments at two weeks of age (Table 7.1, 7.2, 7.3, and 7.4).

Table 7.1 Fatty Acid Composition of GM1a in the SPM of Rats Fed Low LNA, Control, or AA + DHA Diet at Two Weeks of Age*

	Low LNA[T]	Control[†]	AA+DHA[‡]
Fatty acid (% wt/wt)			
C12:0	1.04	0.14	0.42
C14:0	4.42	2.48	4.10
C16:0	52.2	43.9	44.9
C18:0	18.7	14.4	24.2
C20:0	2.75	0.69	0.86
C22:0	0.50	0.35	0.39
C24:0	0.97	0.67	0.73
C18:1n-9	5.94	11.5	5.14
C18:1n-7	1.20	2.64	1.13
C20:1n-9	0.72	0.13	0.04
C22:1n-9	0.35	0.09	0.10
C24:1n-9	0.29	0.02	2.12
C18:2n-6	0.22	10.5	9.77
C20:4n-6	6.82	6.47	2.48
C18:3n-3	0.87	0.95	0.94
C22:6n-3	2.97	5.12	2.65
Σ Sat	80.6	62.5	75.6
Σ Mono	8.49	14.41	8.53
Σ N-6	7.04	17.0	12.3
Σ N-3	3.84	6.08	3.59

[*] n=1 (36 pooled brains) for each experimental diet.

[T] Low LNA was obtained by the addition of safflower oil as the fat blend.

[†] The control fat diet of LA to LNA of 7.1:1 approximates the fatty acid composition used in SMA® infant formula.

[‡] The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

nd, not detected.

Table 7.2 Fatty Acid Composition of GD1a in the SPM of Rats Fed Low LNA, Control, or AA + DHA Diet at Two Weeks of Age[*]

	Low LNA[T]	Control[†]	AA+DHA[‡]
Fatty acid (% wt/wt)			
C12:0	1.10	0.17	4.21
C14:0	5.07	2.34	4.15
C16:0	54.0	42.6	39.5
C18:0	13.5	17.6	23.1
C20:0	2.68	1.19	0.62
C22:0	0.51	0.36	0.31
C24:0	nd	0.54	0.30
C18:1n-9	7.80	9.57	8.73
C18:1n-7	1.52	2.07	1.71
C20:1n-9	0.49	0.20	0.36
C22:1n-9	0.21	0.37	nd
C24:1n-9	nd	0.16	nd
C18:2n-6	6.78	9.29	8.83
C20:4n-6	4.54	7.77	5.01
C18:3n-3	0.43	0.93	0.73
C22:6n-3	1.38	4.89	2.42
Σ Sat	76.8	64.8	72.2
Σ Mono	10.0	12.4	10.8
Σ N-6	11.3	17.1	13.8
Σ N-3	1.81	5.82	3.15

[*] n=1 (36 pooled brains) for each experimental diet.

[T] Low LNA was obtained by the addition of safflower oil as the fat blend.

[†] The control fat diet of LA to LNA of 7.1:1 approximates the fatty acid composition used in SMA® infant formula.

[‡] The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

nd, not detected.

Table 7.3 Fatty Acid Composition of GD1b in the SPM of Rats Fed Low LNA, Control, or AA + DHA Diet at Two Weeks of Age[*]

	Low LNA[T]	Control[†]	AA+DHA[‡]
Fatty acid (% wt/wt)			
C12:0	0.65	11.0	0.61
C14:0	3.31	12.0	2.37
C16:0	34.0	33.8	14.1
C18:0	36.2	27.3	65.1
C20:0	4.38	1.34	2.11
C22:0	1.11	0.25	0.22
C24:0	1.55	1.62	0.57
C18:1n-9	6.20	3.87	2.95
C18:1n-7	1.10	0.54	0.52
C20:1n-9	2.55	0.26	0.39
C22:1n-9	0.77	0.61	0.39
C24:1n-9	0.14	2.86	5.01
C18:2n-6	4.02	2.27	3.03
C20:4n-6	1.97	0.59	0.42
C18:3n-3	0.28	0.94	1.85
C22:6n-3	1.79	0.77	0.34
Σ Sat	81.2	87.3	85.1
Σ Mono	10.8	8.14	9.26
Σ N-6	5.99	2.86	3.45
Σ N-3	2.07	1.71	2.19

[*] n=1 (36 pooled brains) for each experimental diet.

[T] Low LNA was obtained by the addition of safflower oil as the fat blend.

[†] The control fat diet of LA to LNA of 7.1:1 approximates the fatty acid composition used in SMA® infant formula.

[‡] The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

nd, not detected.

Table 7.4 Fatty Acid Composition of GT1b in the SPM of Rats Fed Low LNA, Control, or AA + DHA Diet at Two Weeks of Age*

	Low LNA[T]	Control[†]	AA+DHA[‡]
Fatty acid (% wt/wt)			
C12:0	0.85	0.46	0.56
C14:0	3.26	2.61	2.45
C16:0	41.9	27.4	14.2
C18:0	34.9	49.4	70.5
C20:0	3.81	1.39	2.39
C22:0	0.49	0.47	nd
C24:0	nd	0.43	nd
C18:1n-9	6.17	5.07	2.82
C18:1n-7	0.97	0.81	nd
C20:1n-9	0.90	0.50	0.69
C22:1n-9	0.68	1.27	nd
C24:1n-9	nd	0.26	nd
C18:2n-6	3.89	6.16	5.24
C20:4n-6	1.52	2.11	0.64
C18:3n-3	0.71	0.64	0.51
C22:6n-3	nd	1.02	nd
Σ Sat	85.2	82.2	90.1
Σ Mono	8.71	7.91	3.51
Σ N-6	5.41	8.27	5.88
Σ N-3	0.71	1.65	0.51

[*] n=1 (36 pooled brains) for each experimental diet.

[T] Low LNA was obtained by the addition of safflower oil as the fat blend.

[†] The control fat diet of LA to LNA of 7.1:1 approximates the fatty acid composition used in SMA® infant formula.

[‡] The AA + DHA diet was obtained by the addition of ARASCO™ and DHASCO™ oil to the control diet fat blend.

nd, not detected.

A. DISCUSSION

The results obtained from this study demonstrate that C20:4n-6 and C22:6n-3 are present in SPM gangliosides and dietary fat can alter the C20:4n-6 and C22:6n-3 content of SPM gangliosides.

The SPM gangliosides studied had one characteristic in common: the predominant fatty acid was C16:0 and C18:0. This finding is in agreement with other studies which showed that saturated fatty acids, particularly, C16:0 and C18:0 are abundant in gangliosides (Berra & Galli, 1971; Vanier et al., 1973; Svennerholm et al., 1991 & 1994). Analysis of the fatty acid composition of SPM gangliosides by GC-MS revealed that C20:4n-6 and C22:6n-3 were present. The presence of C20:4n-6 and C22:6n-3 in SPM gangliosides was unexpected since previous studies on the fatty acid composition of rat SPM gangliosides did not detect PUFAs. Avrova (1986) has also shown that C20:4n-6 and C22:6n-3 are present as the fatty acyl moiety of brain gangliosides in some species of fish. It was found in fish that C20:4n-6 and C22:6n-3 constituted up to 5 and 25 % of the total brain ganglioside fatty acids, respectively. This is the first study, to the author's knowledge, that has shown the presence of C20:4n-6 and C22:6n-3 in the fatty acyl moiety of rat SPM gangliosides.

The data suggests that the C20:4n-6 and C22:6n-3 content of rat SPM gangliosides may be altered by dietary fat. However, a previous study by Berra et al. (1976) showed in whole brain of rats from birth to 100 days of age that alterations in diet fat altered the amount but not the fatty acid composition of individual gangliosides. The reason for the differences between the two studies could be due to the diet treatments, amount of diet fat, or SPM vs. whole brain studied. The mechanism for the diet effect on the C20:4n-6 and C22:6n-3 of SPM gangliosides may be due to the activity and specificity deacylation-reacylation enzymes for fatty acids of gangliosides similar to that observed with phospholipids (Saito & Saito, 1991). Moreover, there may be a common pool of fatty-acyl CoA or ceramides that are altered by diet fat and used for synthesis of sphingolipids (Saito & Saito, 1991).

The presence of C20:4n-6 and C22:6n-3 in SPM gangliosides and the effect of diet fat on their fatty acid composition could effect the fluidity of SPM and the calcium flux involved in synaptic transmission. It has been proposed that the reversible binding of calcium to the sialic acid residue of gangliosides at the SPM is involved in the mechanism of synaptic transmission (reviewed by Rahmann et al., 1982 and Wu & Ledeen, 1994). The release of calcium from the ganglioside-calcium complexes may locally change the fluidity of SPM leading to neurotransmitter release. Thus, dietary fat induced alterations in the SPM C20:4n-6 and C22:6n-3 content of ganglioside could alter the binding of calcium to the sialic acid residues of gangliosides, and therefore modulate synaptic transmission.

In conclusion, the data from this study in rats at two weeks of age show the presence of C20:4n-6 and C22:6n-3 in SPM ganglioside and suggest that dietary fat may induce changes in the C20:4n-6 and C22:6n-3 content of SPM gangliosides.

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CHAPTER VIII. THE PRESENCE OF ALKALINE CERAMIDASE IN THE SYNAPTIC PLASMA MEMBRANE OF TWO-WEEK-OLD RAT PUPS

A. INTRODUCTION

Sphingolipids are complex lipids that play a structural role in cell membranes (Coroneos, 1995). Sphingolipids consist of a long-chain base (sphingosine), an amide-linked fatty acyl groups, usually saturated, and a polar or glycosidic head group (Hannun, 1996). Recently, derivatives of sphingolipid metabolism have been identified as membrane signal transduction molecules (Hannun, 1996; Hannun & Obeid, 1997). SM, the major membrane sphingolipid, can be hydrolyzed by SMase to form ceramide, a secondary messenger which stimulates cell differentiation, inhibits proliferation and has been associated with apoptosis (Jarvis et al., 1994; Jayadev et al., 1995). CDase (EC 3.5.1.23) is widely distributed in animal tissues such as brain and catalyzes hydrolysis of ceramide to yield sphingosine and a fatty acid (reviewed by Hassler & Bell, 1993). CDase is important to animal tissues because it is the only known mechanism for ceramide catabolism (Tani et al., 2000). Sphingosine is also known to affect a variety of biological functions (Ballou, 1992). There are three distinct isoforms of CDase (acidic, neutral, and alkaline) that have different pH optima, 4.0, 7.6, and 9.0, respectively. The acidic lysosomal CDase has been purified to apparent homogeneity (Bernardo et al., 1995) and characterized (Li et al., 1998). Neutral CDase activity were found in porcine intestine (Nilsson, 1969; Nilsson & Duan, 1999), guinea pig skin (Yada et al., 1995) and human fibroblasts (Morell & Braun, 1972) and recently purified and cloned from mouse liver (Tani et al., 2000). The alkaline CDase was described in human cerebellum (Sugita et al., 1972), fibroblasts (Momoi et al., 1982) and in many rat tissues (Spence et al., 1985). A non-lysosomal CDase from rat brain was recently purified and characterized (El Bawab et al., 1999). However, to date, no study has determined the presence of alkaline CDase in SPM. Based on the evidence that ceramide and sphingosine play an important role in biological processes and ceramide constitutes the core structure of several sphingolipids which are highly abundant in SPM, it would be of interest to determine whether alkaline CDase activity is present in the SPM. The aim of the present study was to determine if the SPM of rat pups at two-weeks of age has alkaline CDase.

B. MATERIALS AND METHODS

1. Animal Care

Breeding of Sprague-Dawley rats have been described in **Chapter III**. Rats sacrificed at two weeks of age received only maternal milk. One entire litter of rat pups fed the same diet was sexed and weighed before decapitation. Excised brains were placed in ice-cold 0.32 M sucrose. Six brains from the same sex were pooled per sample for isolation of SPM. Three litters per diet were used.

2. Diets

The diet contained 20% (wt/wt) fat and met all essential nutrient requirements (Clandinin & Yamashiro, 1980). The control fat treatment was formulated to approximate the fatty acid composition of an existing infant formula providing an C18:2n-6 to C18:3n-3 fatty acid ratio of 7.8:1 (Table 3.1; **Chapter III**).

1. Synaptic Plasma Membrane Isolation

SPMs from rat pups at two-weeks of age were isolated as described in **Chapter VI**. The SPM purity was tested as described in **Chapter VI**.

2. Ceramide Synthesis

The ^{3}H-palmitoyl ceramide was prepared as follows (Kishimoto, 1975): to a mixture of 34 μmol cold palmitic acid, 75 μCi (9, 10-^{3}H) palmitic acid (30 – 60 Ci/mmol; New England Nuclear-DuPont, Boston, MA), 102 μmol triphenylphosphine (Sigma Chemical Co., St. Louis, MO), and 102 μmol 2,2'-dipyridyldisulfide (Sigma Chemical Co., St. Louis, MO) was added 0.4 mL of methylene chloride (Sigma Chemical Co., St. Louis, MO) solution containing 34 μmol sphingosine (free base; Sigma Chemical Co., St. Louis, MO). This mixture was stirred at room temperature for 5 hr. The mixture became yellow immediately after mixing. Solvent was removed from the reaction mixture by evaporation under nitrogen and the residue was resuspended in 500 μL of chloroform : methanol (2:1, by vol.). The ceramides synthesized were isolated and purified on a silica gel "G" plate (20 x 20 cm; Analtech, Newark, DE, USA) developed in a solvent system of chloroform : methanol : acetic acid (90:2:8, by vol.). The plate was sprayed with 0.01% (wt/vol.) primulin in water. Purified ceramide was located under ultraviolet light by comparison with ceramide standard (Sigma Chemical Co., St. Louis, MO), and eluted with chloroform : methanol (2:1, by vol.). An aliquot of the chloroform : methanol solution was added to a liquid scintillation cocktail (ScintiSafe™, Fisher Scientific, Fair-Lawn, NJ, USA) and taken for liquid scintillation counting (Beckman LS 5801). Counting efficiency was approximately 97% and all counts were corrected for counting efficiency. The ceramides were stable to alkaline hydrolysis (0.5 N KOH in methanol at 25 °C for 12 hr) under conditions where all fatty acid esters were released from total phospholipids.

5. Ceramidase Assay

Alkaline CDase was assayed by measuring the amount of radioactive palmitic acid released from ^{3}H-palmitoyl ceramide as described by Nikolova-Karakashian & Merrill (1999). The ceramide suspension was prepared by adding 10 mg of Triton X-100 (Sigma Chemical Co., St. Louis, MO) and 16 mg of sodium cholate (Sigma Chemical Co., St. Louis, MO) in chloroform : methanol (2:1, by vol.) to 1 mL of labeled ^{3}H-palmitoyl ceramide in chloroform : methanol (2:1). The mixture was vortexed and evaporated to dryness under nitrogen. The residue was suspended in 1 mL of 0.25 M sucrose – 1mM EDTA and the tubes were placed in a sonifying water bath at 37°C for 10 min. The final suspension was free of particulates and was opalescent. This suspension containing 0.25 mg Triton X-100, 0.40 mg sodium cholate, and 50 μmol of ^{3}H-palmitoyl ceramide in 25 μL solution was stored at 4 °C.

The reaction mixture consisted of 50 μmol ^{3}H-palmitoyl ceramide (20 μCi/μmol) solution, 250 – 500 μg of SPM protein, and Hepes buffer (pH 8.0) in a final volume of 200 μL. After incubation in a shaking water bath at 37 °C for 1 hr, the reaction was stopped by adding 1 mL of chloroform : methanol (2:1, by vol.) containing 50 μg of carrier palmitic acid. Tubes were vigorously mixed and centrifuged at 2000 g for 15 min. The lower phase was spotted on a silica gel "G" plate (20 x 20 cm) and developed in a solvent system of chloroform : methanol : acetic acid (94:1:5, by vol.). The free fatty acid band was located by using iodine vapor, and the corresponding areas were scraped from the plate into a scintillation vial with liquid scintillation cocktail (Scintisafe™, Fisher Scientific). The radioactivity of the free fatty acid released was counted in a liquid scintillation counter (Beckman LS 5801). CDase activity after correction for blank value (boiled SPM protein in incubation mixture) was expressed as ρmol ^{3}H-palmitic acid/mg·hr.

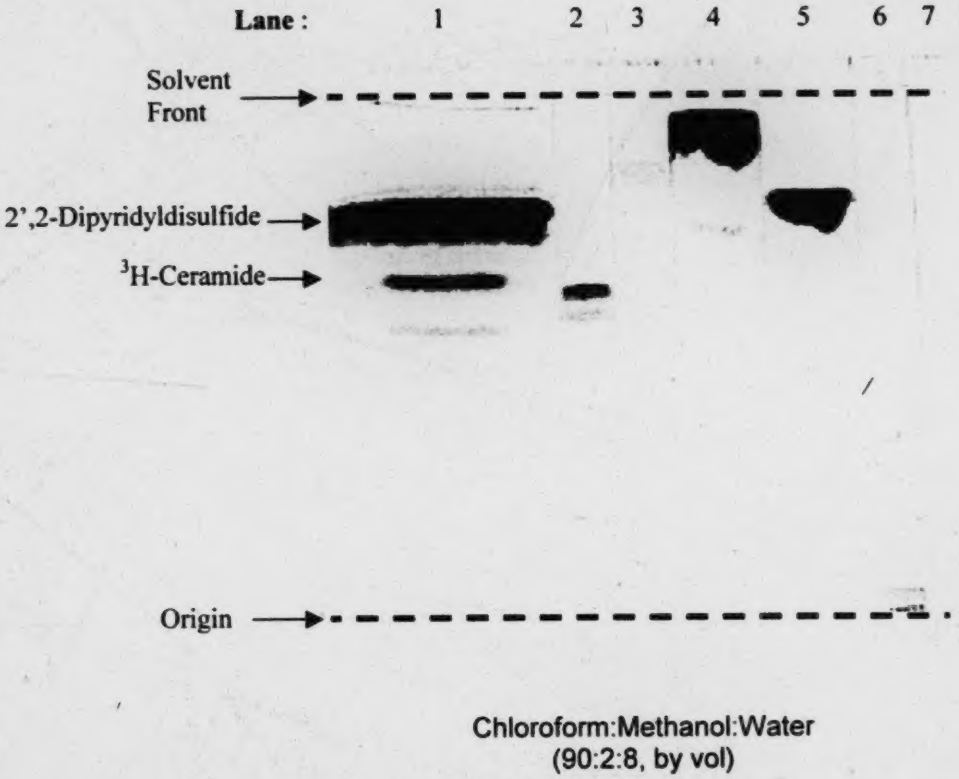
C. RESULTS

1. Purity of Synaptic Plasma Membrane Preparation

The purity of the SPM preparation has been described previously (Hargreaves & Clandinin, 1987). The SPM preparations contained only minor cross-contamination (<5%) from mitochondria as determined by RNA and succinate dehydrogenase analysis (data not shown). The SPM marker, Na, K-ATPase, was approximately 8-fold greater in the SPM (33.8 ± 3.0 μmol Pi/mg/hr) compared to the brain homogenate (4.1 ± 1.2 μmol Pi/mg/hr).

2. Purity of ^{3}H-Palmitoyl Ceramide

^{3}H-Palmitoyl ceramide was synthesized after 5 hr at room temperature as indicated by thin-layer chromatography analysis of the reaction mixture (Figure 8.1). The ^{3}H-palmitoyl ceramide product was isolated with an overall yield of approximately 70%, which is similar to that shown by Kishimoto (1975).



Lane 1: Test sample (^{3}H-palmitoyl-ceramide synthesized in vitro)
Lane 2: Ceramide standard (C16:0 and C24:1 ceramide)
Lane 3: Palmitic Acid
Lane 4: Triphenylphosphine
Lane 5: 2',2-Dipyridyldisulfide
Lane 6: Sphingosine (free base)
Lane 7: Blank (no sample)

Figure 8.1 Separation of Synthesized ^{3}H-Palmitoyl Ceramide on Thin-Layer Chromatography Plate.

Table 8.1 Alkaline CDase Activity of Rat Pups on a Control Diet at Two Weeks of Age*

Brain homogenate alkaline CDase activity: 49.6 ± 11.7 ρmol ^{3}H-palmitic acid / mg / hr

SPM alkaline CDase activity: 745 ± 160 ρmol ^{3}H-palmitic acid / mg / hr

Enrichment: $$\frac{\text{SPM alkaline CDase activity}}{\text{Brain homogenate alkaline CDase activity}} = 15.0$$

* Values are mean ± SEM for n=6.

D. DISCUSSION

In the present study, alkaline CDase activity was detected in the SPM of two-week-old rat pups. The CDase activity in SPM was 15-fold greater than in the brain homogenate (Table 8.1). The activity of CDase in the SPM was lower than that reported by Spence et al. (1986) in rat cerebrum, cerebellum, and brain stem. The reason for the lower alkaline CDase in SPM in the present study compared to brain cerebrum, cerebellum, and brain stem homogenates may be due to the different age, strain, or diet treatment of the rats studied. In the study by Spence et al. (1986), the age and diet treatment is unknown. Although neutral and alkaline CDase has been detected in microsomes of rat liver (Stoffel & Melzner, 1980), there is no contamination of microsomes in the SPM since the membrane preparation was devoid of RNA.

^{3}H-Palmitoyl ceramide was used to assay alkaline CDase activity in the SPM because palmitic, as well as, stearic acid are the predominant fatty acids associated with the ceramide moiety of sphingolipids. Also, comparison of brain alkaline CDase activities from the present study could be made to Spence et al. (1986) study.

Ceramide is involved as structural and functional components of sphingolipids (Hannun, 1996). Ceramide plays an important role as an intracellular signaling molecule (reviewed by Hannun et al., 1993; Mathias & Kolesnick, 1993) and enzymes, such as, CDase can regulate the metabolism of ceramide and consequently ceramide mediated functions (Mao et al., 2000; Tani et al., 2000).

In conclusion, the present study is the first to identify alkaline CDase activity in the SPM of two-week-old rat pups. CDase is thought to be rate limiting enzyme in the production of sphingosine and sphingosine-1-phosphate because sphingosine is only generated from ceramide by the action of CDase and not by de novo synthesis (Tani et al., 2000). Hence, alkaline CDase could play a critical role in signal transduction mediated by sphingosine and sphingosine-1-phosphate.

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CHAPTER IX. GENERAL SUMMARY, CONCLUSIONS, LIMITATIONS, IMPLICATIONS, AND FUTURE RESEARCH

A. GENERAL SUMMARY

The hypotheses (**Chapter II**) tested in this thesis have been verified as follows:

Hypothesis 1.

It was hypothesized that feeding a high C18:3n-3 diet will not increase the C22:6n-3 content in neuronal cell phospholipids from whole brain of rats at two weeks of age. The results obtained suggest that raising the dietary C18:3n-3 content is inadequate to increase the C22:6n-3 content of neuronal cell phospholipids of two-week-old rat pups.

Hypothesis 2.

It was hypothesized that feeding a high C18:3n-3 diet will increase the C18:3n-3 content in whole body and tissue lipids of rats at two weeks of age. The results obtained suggest that the metabolic fate of high C18:3n-3 diet is deposition of C18:3n-3 in adipose tissue and skin (epidermis, dermis, and subcutaneous tissue) of two-week-old rat pups.

Hypothesis 3.

It was hypothesized that feeding diets with preformed C22:6n-3 will increase the content of C22:6n-3 in neuronal and glial cell membrane phospholipids from whole brain of rats at two weeks of age. The results obtained suggest that preformed C22:6n-3 significantly increases the C22:6n-3 content in PE and PS of neuronal and glial cell phospholipids from two-week-old rat pups.

Hypothesis 4.

It was hypothesized that feeding a diet with C20:4n-6 and C22:6n-3 will increase the total and individual phospholipids and cholesterol content of the SPM of rats at two and five weeks of age. The results obtained suggest that diets with C20:4n-6 and C22:6n-3 does not alter the SPM total and individual phospholipid content and cholesterol content of rats at two and five weeks of age.

Hypothesis 5.

It was hypothesized that feeding a diet with C20:4n-6 and C22:6n-3 will increase the C20:4n-6 and C22:6n-3 content in total and individual phospholipids of the SPM of rats at two and five weeks of age. The results obtained suggest that dietary C20:4n-6 and C22:6n-3 increases the C20:4n-6 and C22:6n-3 content of SPM total and individual phospholipids of rats at two and five weeks of age.

Hypothesis 6.

It was hypothesized that the Km and Vmax of Na, K-ATPase in the SPM of rats will increase from two to five weeks of age. The results obtained suggest that Na, K-ATPase Km is decreased and Vmax is increased from two to five weeks of age.

Hypothesis 7.

It was hypothesized that feeding diet with C20:4n-6 and C22:6n-3 will not alter the Km but increase the Vmax of Na, K-ATPase in the SPM of rats at two and five weeks of age. The results obtained suggest that dietary C20:4n-6 and C22:6n-3 does not alter the Km but increases the Vmax of SPM Na, K-ATPase of rat pups of two and five weeks of age.

Hypothesis 8.

It was hypothesized that C20:4n-6 and C22:6n-3 were present in SPM gangliosides and feeding a dietary fat will alter the C20:4n-6 and C22:6n-3 content of individual gangliosides in the SPM of rats at two weeks of age. The results obtained demonstrate that C20:4n-6 and C22:6n-3 are present in SPM ganglioside and dietary fat may alter the C20:4n-6 and C22:6n-3 content of individual gangliosides in the SPM of two-week-old rat pups.

Hypothesis 9.

It was hypothesized that alkaline CDase is present in the SPM of rat pups at two weeks of age. The results obtained demonstrate that alkaline CDase is present in SPM of rat pups at two weeks of age.

B. CONCLUSIONS

The data presented in this thesis expands on existing knowledge of the effects of dietary fat on membrane lipid composition and function in brain. Feeding diets with or without C18:3n-3 or C20:4n-6 and C22:6n-3 has been shown in this thesis to play an important role in brain structure and function. The important concepts that this thesis has contributed are as follows:

1. High dietary C18:3n-3 content does not significantly increase the C22:6n-3 content in neuronal cells from whole brains of rats at two weeks of age.
2. High dietary C18:3n-3 significantly increases the C18:3n-3 but not the C22:6n-3 content of whole body and tissue lipids with the major deposition site being the skin including the subcutaneous fat of rats at two weeks of age.
3. Dietary C22:6n-3 is more effective than high levels of C18:3n-3 at significantly increasing the C22:6n-3 content in neuronal and glial cell phospholipids, particularly, PE and PS of rats at two weeks of age.
4. Dietary C20:4n-6 and C22:6n-3 does not increase the cholesterol, total and individual phospholipid content of SPM of rats at two and five weeks of age.
5. Dietary C20:4n-6 and C22:6n-3 increases the C20:4n-6 and C22:6n-3 content of rat total and individual phospholipids at two and five weeks of age.
6. The Vmax and Km of Na, K-ATPase in the rat SPM significantly increases during development from two to five weeks of age.
7. Dietary C20:4n-6 and C22:6n-3 compared to low C18:3n-3 diet significantly increases the Vmax but does not alter the Km of Na, K-ATPase in the rat SPM at two and five weeks of age.
8. C20:4n-6 and C22:6n-3 are present and feeding a dietary fat may alter the C20:4n-6 and C22:6n-3 content of individual gangliosides in the rat SPM at two weeks of age.
9. Alkaline CDase is present in the SPM of rat pups at two weeks of age.

C. LIMITATIONS

The studies presented in this thesis employed maternal dietary fat alterations as a method of delivering various fatty acid compositions to rapidly growing rat pups. A limitation of this maternal feeding model is that it hinders the ability to directly manipulate the fatty acid composition of the nutrient supply to the rat pups during which most of the brain growth spurt is occurring in the first three postnatal weeks of life (Dobbing and Sands, 1979). Therefore, in this model there is the confounding influence of LCPUFAs, such as C20:4n-6 and C22:6n-3, present in the stomach contents (dams' milk) of rat pups. In addition, the maternal milk composition in this model can have changes that are secondary to the changes in dietary n-6 and / or n-3 fatty acids (i.e. hormones, cytokines, and growth factors) (Ward et al., 1996).

The artificial rearing (AR) model in the rat pups would have been ideal to use in the studies presented in this thesis. In this model, rat pups are removed from their dams and fed, via gastrostomy tube, a rat milk substitute which closely resembles the composition of rat milk, and whereby the fatty acid composition can be altered experimentally (Patel and Hiremagalar, 1992). This AR model permits the evaluation of dietary fat treatments on brain development without the confounding influence of C20:4n-6 and C22:6n-3 present in the maternal diet and mammary processes (Patel and Hiremagalar, 1992). Furthermore, the AR model approximates the extremely premature infant who is exposed to the stress of maternal separation, nasogastric formula feeding, testing, and surgical procedures (Ward et al, 1996). However, this model is very labor intensive and expensive.

D. IMPLICATIONS OF THESIS

The findings from this thesis in rats have important implications for neonatal feeding. Infant formulas currently marketed in North America are devoid of C20:4n-6 and C22:6n-3. The only n-3 fatty acid present in these formulas is C18:3n-3. It has been questioned whether C18:3n-3 in infant formulas can meet the C22:6n-3 requirements for brain growth and development. The findings from this thesis demonstrate in rats that raising the dietary C18:3n-3 content does not significantly raise the C22:6n-3 content of brain phospholipids. In fact, this thesis has shown that the metabolic fate of high dietary C18:3n-3 is deposition of C18:3n-3 in rat adipose and subcutaneous tissues especially skin. Furthermore, dietary C22:6n-3 was shown to be more effective than low or high levels of C18:3n-3 at increasing the C22:6n-3 content of rat brain phospholipids. Therefore, these results suggest that formulas providing only C18:3n-3 may not meet the C22:6n-3 requirements of neonatal brain.

The functional importance of C22:6n-3 in rat brain was demonstrated by showing that the low SPM C22:6n-3 phospholipid content is associated with low SPM Na, K-ATPase activity. Therefore, these findings suggest that infants with lower levels of C22:6n-3 in SPM phospholipids may have low SPM Na, K-ATPase activity that will ultimately affect synaptic transmission in infant brain.

The findings from this thesis indicated that rat SPM gangliosides contain C20:4n-6 and C22:6n-3 and the content of C20:4n-6 and C22:6n-3 in SPM gangliosides may be altered by dietary fat. These results if extrapolated to human neonates would suggest that infants fed formulas containing C20:4n-6 and C22:6n-3 incorporate these fatty acids into the fatty acyl moiety of SPM gangliosides.

The presence of alkaline CDase activity in rat SPM is novel. This finding implies that neonates possess SPM alkaline CDase activity. The presence of alkaline CDase in neonatal SPM may be significant since sphingolipids are involved in mediating biological responses. Hence, it is conceivable that SPM alkaline CDase in infants may play an important role in brain functions.

E. FUTURE RESEARCH

Progress has been made over the last decade in understanding the desaturation and chain elongation of essential fatty acids and the influence of these fatty acids and their long-chain homologues on membrane fatty acid composition and function. However, as new information is gathered on the mechanism, regulation, and function of fatty acid desaturation and elongation reactions in mostly animal models, the extent to which this information applies to human infants needs to be fully elucidated. The application of cloning techniques and molecular probe technologies to desaturation and elongation enzymes are beginning to be used but much more work is needed. Specific probes for the delta-6 desaturase will be beneficial for determining whether this rate-limiting enzyme(s) acting on C18:2n-6, C18:3n-3, C24:4n-6, and C24:5n-3 are identical. Specific studies on the modulation of the desaturase enzymes should provide insight into regulation at the genomic level. The extent to which desaturase and elongase are regulated and if the sequence of reactions catalyzed by these enzymes are physically associated to provide "channeled" conversion to their major end products awaits further research. The subcellular location of these enzymes to other compartments beside the endoplasmic reticulum like peroxisomes or nuclear and plasma membrane needs further work.

The role and function of C18:3n-3 in the skin of rats needs to be understood. Further studies need to determine if C18:3n-3 present in the skin may be synthesized to other fatty acids, such as, C16:0, C18:0, and C18:1n-9, or cholesterol as observed in brain of suckling rats. Studies are also needed to determine whether the C18:3n-3 in skin is preferentially β-oxidized and used as a source of energy rather than for synthesis of n-3 long-chain polyenes like C20:5n-3 and C22:6n-3. The relevance of C18:3n-3 in skin of rats to human infants remains to be determined.

Future studies to determine the mechanism for the increased activity of Na, K-ATPase observed with the control and AA + DHA compared to the low LNA diet are needed. It is conceivable that the increased activity of Na, K-ATPase with the control or AA+ DHA diet may be due to decreased degradation rate, or increased synthesis rate, or both processes working in concert. Hence, molecular biology techniques examining Na, K-ATPase protein and gene expression will be insightful.

Future research is needed to determine the exact role of diet fat on cholesterol and PUFA content in modulating the SPM Na, K-ATPase kinetics. It is possible that dietary fat affects the distribution of cholesterol in the exofacial and cytofacial leaflet thereby altering the fluidity of the two leaflets and thus Na, K-ATPase activity.

C20:4n-6 and C22:6n-3 in SPM may directly affect Na, K-ATPase kinetics by its presence as the fatty acid moiety by its presence as the fatty acid moiety of annular membrane phospholipids or indirectly via modulation of eicosanoids that can affect Na, K-ATPase activity. Further investigations should clarify or provide further information to the exact mechanism of C20:4n-6 and C22:6n-3 on Na, K-ATPase kinetics.

Despite the tremendous progress over the last decade in understanding the cellular function of sphingolipids, this area is still in its infancy. The exact physiological roles which gangliosides play in biological processes must be studied further. Why are there so many different molecular species of gangliosides ? What role does C20:4n-6 and C22:6n-3 play in gangliosides ? Is the subtle changes induced by diet fat in the ceramide moiety of gangliosides to optimize its physical properties or to achieve a greater specificity in ganglioside-protein interaction; or are gangliosides synthesized for other reasons, such as, signal transduction ? Future work should provide answers to these questions.

The precise metabolic and biological roles of alkaline CDase are not entirely understood. *In vitro* catalytic activity and metabolic studies suggest that ceramide catabolism is their primary biological role. The alkaline CDase may be responsible for the recycling of sphingosine from the cell sphingolipids. Moreover, alkaline CDase could be used to salvage unused ceramides synthesized for sphingolipids. Conceivably, alkaline CDase may also perform the task of regulating the cellular levels of sphingosine or ceramide that can be used as cellular effectors. Finally, alkaline CDases in the SPM may function to regulate the types and levels of particular fatty acids, possibly PUFAs, incorporated into ceramides, and consequently its overall sphingolipid structure and function. The potential importance of alkaline CDase in producing sphingosine for a regulatory purpose within cells makes them prime candidates for future study. Some future research should be to characterize alkaline CDase activity with respect to dietary fat alterations and biological functions.

The studies in this thesis were performed on Sprague-Dawley rats. It would be of interest to study the effects of dietary fat on brain C20:4n-6 and C22:6n-3 content in an animal model of PKU. Recently, a true mouse model of PKU with a deficiency of phenylalanine hydroxylase (PAH) and a similar pathology to human PKU has been developed (Shedlovsky et al., 1993). This mouse strain generated through mutagenesis of chromosome 10, the PAH gene, by ethylnitrosurea, closely simulates human PKU phenotype (McDonald, 1994). In this disease, there are low levels of brain C20:4n-6 and C22:6n-3 partly due to the absence C20:4n-6 and C22:6n-3 in PKU formulas and the low

C20:4n-6 and C22:6n-3 content in the diet of individuals with PKU (Agostoni et al., 1995; Poge et al., 1998). PKU formulas are used in place of or in addition to human milk for infants born with PKU (Greve et al., 1994). Most of the commercial PKU formulas contain casein hydrosylate to which some vegetable oils like soy, coconut, palm, peanut, or corn oil are added as the only fat component. These formulas contain only vegetable oils with C18:2n-6 and C18:3n-3 as the only source of n-6 and n-3 fatty acids, respectively. None of the PKU formulas on the market contain preformed C20:4n-6 and C22:6n-3. The PKU diet is devoid of meat and fish, which contain large amounts of C20:4n-6 and C22:6n-3 (Agostoni et al., 1995; Poge et al., 1998). Therefore, it is conceivable that diet fat with C20:4n-6 and C22:6n-3 may normalize brain C20:4n-6 and C22:6n-3 levels in brain of individuals with PKU. It is also of interest to study if these changes in C20:4n-6 and C22:6n-3 content of PKU brain phospholipids could have some functional effects, such as alterations in the kinetics of SPM Na, K-ATPase. Therefore, dietary C20:4n-6 and C22:6n-3 may have a profound effect on brain phospholipid fatty acid composition and function, and hence alleviating some of the brain dysfunction in PKU individuals.

The outcome for preterm infants have steadily improved over the past three decades. Advances in neonatal care have resulted in improved survival of infants born as early as 25 weeks of gestation. The provision of effective nutritional support for this vulnerable group has become an area of increasing importance. Optimum nutrition should lead to more normal growth and development with fewer infections or metabolic disturbances. Infant formulas for preterm infants are continuously being refined and the ideal composition is unknown. The studies presented in this thesis show that dietary fat is a strong determinant of brain fatty acid composition and function. The data support the rationale for supplementing infant formulas with preformed C20:4n-6 and C22:6n-3.

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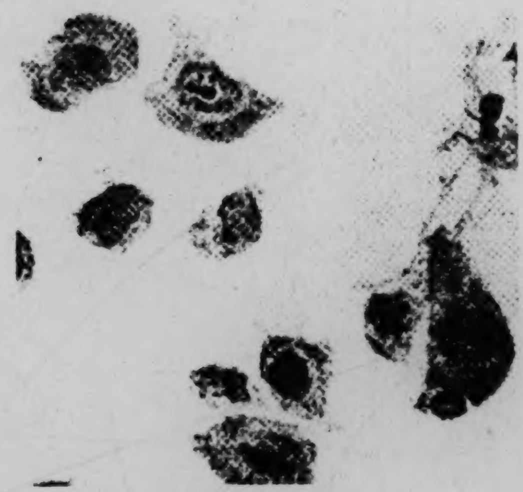
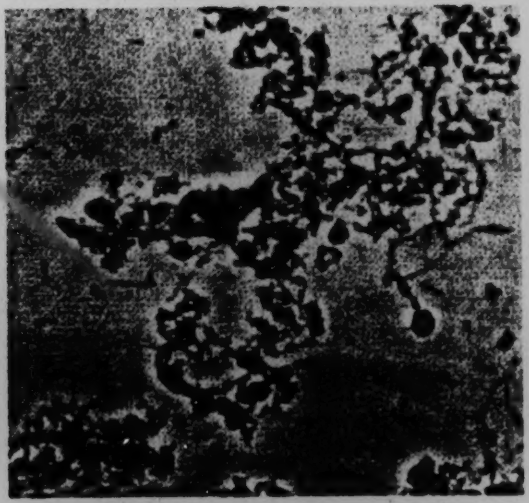
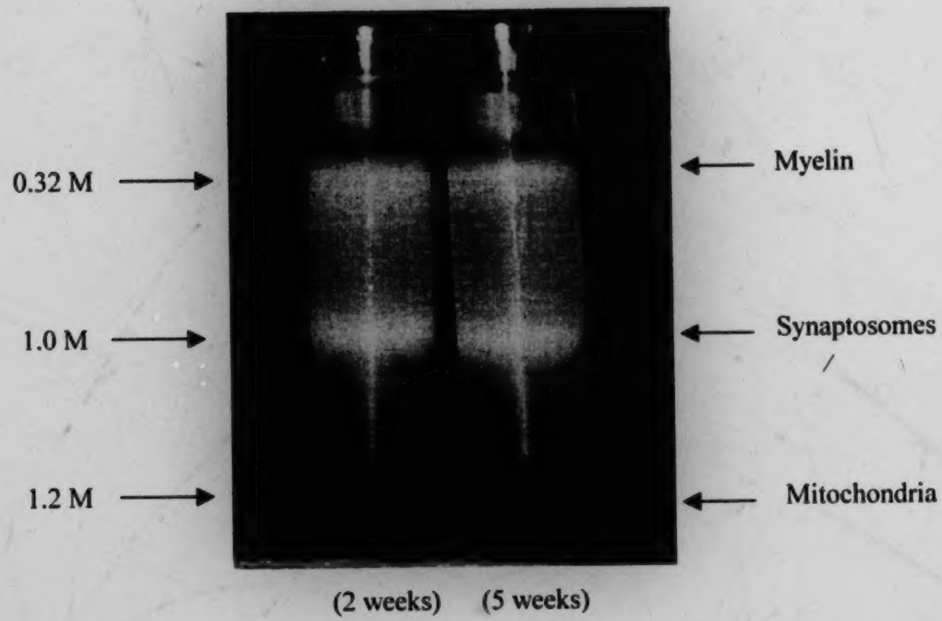


Neuronal Cells



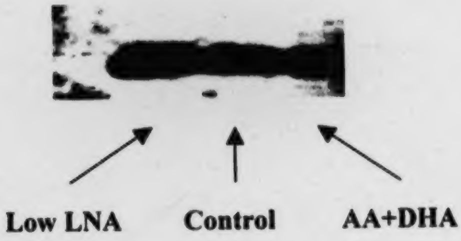
Glial Cells

APPENDIX 1. Neuronal and glial cells isolated from whole brain of rat pups at two weeks of age (1600x) (adapted from Hamberger & Svennerholm, 1971)

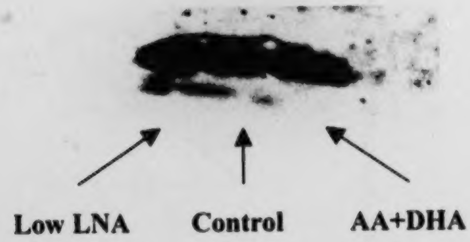


APPENDIX 2. Isolation of synaptosomes on sucrose density gradient from whole brain of rat pups at two and five weeks of age.

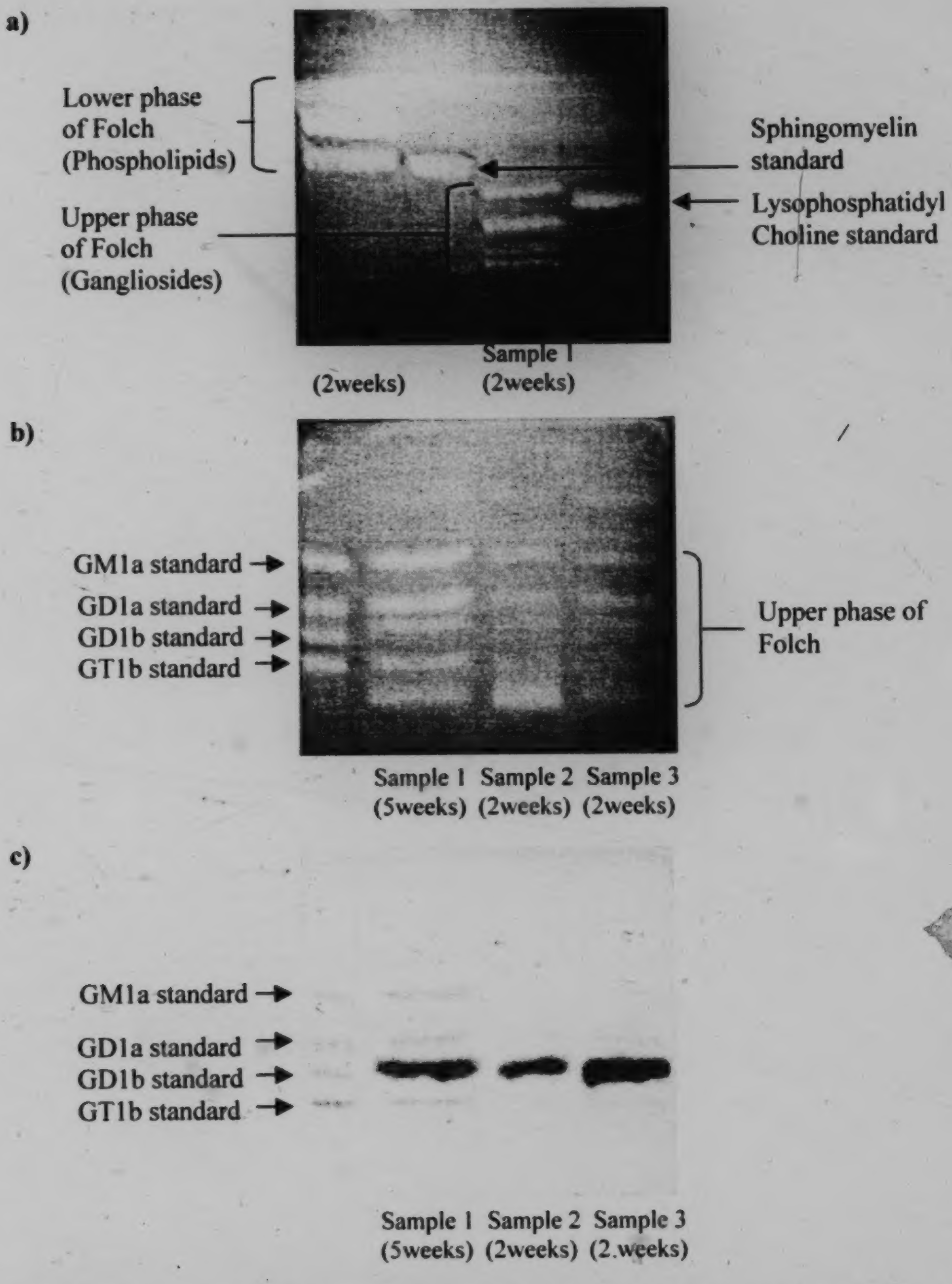
a) **2 Weeks**



b) **5 Weeks**



APPENDIX 3. SDS-PAGE of synaptic plasma membrane Na, K-ATPase β1-subunit of rats fed either low LNA, control, or AA+ DHA diet at a) 2 weeks and b) 5 weeks of age. Gels were probed with monoclonal antibodies specific for β1-subunit of Na, K-ATPase. Each lane contains 20 ug of SPM protein.



APPENDIX 4. Separation of gangliosides from SPM of rats at two and five weeks of age on high performance thin-layer chormatography plates: a) lower and upper phase of Folch extraction of sample sprayed with 0.01% ANSA under ultraviolet light; b) ganglioside standards and upper phase of Folch extraction from samples sprayed with 0.01% ANSA under ultraviolet light; c) ganglioside standards and upper phase of Folch extraction from samples sprayed with resorcinol-HCl.